# Investigating BET proteins as a therapeutic target in keloid scarring

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# Investigating BET proteins as a therapeutic target in keloid scarring.

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# Abstract

Keloid scars form due to an over-exuberant, fibrotic wound response in the skin of predisposed individuals. There is currently no clear genetic link identified, and limited treatment options available. Epigenetic changes in fibrosis, and specifically in keloids, are beginning to be identified and this offers potential targets for treatment.

This project explored the bromodomain and extraterminal (BET) family of proteins: Brd2, Brd3 and Brd4, which function as epigenetic regulators through their binding to acetylated lysine residues, predominantly on histone proteins. We hypothesised that they would be a target of therapeutic value given that their inhibition has shown anti-proliferative and anti-inflammatory properties in other cell types.

We identified an overexpression of Brd2 in keloid tissue compared to normal controls, including a possible expression of a differential Brd2 isoform. We then used a small molecule bromodomain inhibitor, I-BET151 (GSK), to investigate the effect of BET protein inhibition in primary dermal fibroblast cultures from normal skin (NDFs) and keloid scars (KDFs). We confirmed previous literature findings, in other disease models, that BET inhibition decreased proliferation, as well as the expression of the inflammatory cytokine interleukin-6 (IL-6).

In addition, we identified some more novel effects of BET inhibition, including a decrease in fibroblast contraction in a collagen gel model and decreases in proteolytic activity in fibroblast cultures, as well as a more complex *ex vivo* tissue model. Interestingly, we also observed profound differences between NDFs and KDFs in their signalling downstream of IL-6 in serum free environments. In particular, a strong induction of pAkt was observed in KDFs after 30 minutes of cytokine stimulation, which was not seen in NDFs, and BET inhibition abrogated this.

In conclusion, BET inhibition appears to offer therapeutic value in keloid scars by targeting a number of disease-associated cell behaviours, including aberrant signalling responses, which may influence the fibrotic response. Some of these effects may be of relevance in other disease contexts including cancer, where these inhibitors are currently progressing in clinical trials.

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# List of Abbreviations

| | |
|---|---|
| **2/3D** | Two/three dimensional |
| **ALI** | Air-liquid interface |
| **ATP** | Adenosine triphosphate |
| **BCA** | Bicinchoninic assay |
| **BET** | Bromo and extra terminal domain family |
| **Bp** | Base pairs |
| **BRD** | Bromodomain |
| **BSA** | Bovine serum albumin |
| **CBP** | CREB binding protein |
| **cDMEM** | Complete Dulbecco's Modified Eagles Medium (+ 10% FCS, L-glutamine and penicillin streptomycin) |
| **cDNA** | Coding DNA |
| **ChIP** | Chromatin immunoprecipitation |
| **CNS** | Central nervous system |
| **CST** | Cell Signalling Technologies |
| **CTGF** | Connective tissue growth factor |
| **DAB** | 3,3'-diaminobenzidine |
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **DETC** | Dendritic epidermal T cell |
| **DI water** | De-ionised water |
| **DMEM** | Dulbecco's Modified Eagles Medium |
| **DMSO** | Dimethyl Sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **ECL** | Electrochemiluminisence |
| **ECM** | Extracellular matrix |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **EGF** | Epidermal growth factor |
| **ELISA** | Enzyme linked immunosorbent assay |
| **EMT** | Epithelial-mesenchymal transition |
| **EndMT** | Endothelial-mesenchymal transition |
| **FACS** | Fluorescence-activated cell sorting |
| **FCS** | Foetal calf serum |
| **GAPDH** | Glyceraldehyde 3-phosphate dehydrogenase |
| **GSK** | GlaxoSmithKline |
| **GSTT** | Guy's and St Thomas' Trust |
| **GWAS** | Genome-wide Association Study |
| **H&E** | Haematoxylin and eosin |
| **HAT** | Histone acetyl transferase |
| **HBSS** | Hanks buffered saline solution |
| **HDAC** | Histone deacetylase |
| **HLA** | Human leukocyte antigen |
| **HSDF** | Hypertrophic scar dermal fibroblast |
| **HVG** | Haemotoxylin Van Gieson's stain |
| **IF** | Immunofluorescence |

| | |
|---|---|
| **IHC** | Immunohistochemistry |
| **IL** | Interleukin |
| **IMS** | Industrial methylated spirit |
| **IPF** | Idiopathic pulmonary fibrosis |
| **KALT** | Keloid-associated lymphoid tissue |
| **KCL** | King's College London |
| **kDa** | Kilodalton |
| **KDF** | Keloid human dermal fibroblasts |
| **L-glut** | L-glutamine |
| **lncRNA** | Long non-coding RNA |
| **LPS** | Lipopolysaccharide |
| **M1** | Pro-inflammatory/resolution polarised macrophage |
| **M2** | Anti-inflammation, pro fibrotic polarised macrophage |
| **MiR** | MicroRNA |
| **MLL** | Mixed lineage leukaemia |
| **MMP** | Matrix metalloprotease |
| **mRNA** | Messenger RNA |
| **MT-MMP** | Membrane bound matrix metalloproteinase |
| **MTT** | Methylthiazol tetrazolium |
| **NDF** | Normal human dermal fibroblasts |
| **NET** | N extra terminal domain |
| **NLS** | Nuclear localisation signal |
| **P/S** | Penicillin Streptomycin |
| **PBS** | Phosphate buffered saline |
| **PDGF** | Platelet-derived growth factor |
| **PE** | Phycoerythrin |
| **PFA** | Paraformaldehyde |
| **PMA** | Phorbol 12-myristate 13-acetate |
| **Pol** | Polymerase |
| **P-TEFb** | Positive elongation transcription factor b |
| **qPCR** | Quantitative polymerase chain reaction |
| **-r** | Receptor |
| **RA** | Rheumatoid arthritis |
| **RACE** | Rapid amplification cDNA ends |
| **RIN** | RNA Integrity Number |
| **RIPA** | Radioimmunoprecipitation assay buffer |
| **RNA** | Ribonucleic acid |
| **rRNA** | Ribosomal RNA |
| **RT** | Reverse transcription |
| **SDS-PAGE** | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| **SEM** | Standard Error of the Mean |
| **Seq** | Sequencing |
| **SGUL** | St George's University London |
| **SNP** | Single nucleotide polymorphism |
| **SSc** | Systemic sclerosis |

| | |
|---|---|
| **STAT** | Signal transducer and activator of transcription factor |
| **TBS** | Tris buffered saline |
| **TBS-t** | Tris-buffered saline + 0.1% Tween 20 |
| **TGF** | Transforming growth factor |
| **Th** | T helper cell |
| **TIMP** | Tissue inhibitor of matrix |
| **TLR** | Toll-like receptor |
| **TNF** | Tumour necrosis factor |
| **TRIM** | Transcription intermediary factor |
| **UV** | Ultraviolet |
| **VEGF** | Vascular endothelial growth factor |
| **WST** | Water-soluble tetrazolium salt |
| **α-SMA** | Alpha smooth muscle actin |

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# Chapter 1: Introduction

## 1.1. Mammalian skin

### 1.1.1. Anatomy and function of the skin

The skin is a vital integumentary organ protecting the underlying tissue from a range of environmental insults. The skin is derived early in development where a simple ectoderm epithelium overlies mesenchyme. In humans, after several months of foetal development this layer becomes stratified, and pigmented by neural crest derived melanoblasts, and functional appendages can develop (Fuchs, 2007). Fully developed skin from different anatomical locations varies in its composition. Variability in dermal and epidermal thickness, hair follicle and sebaceous gland content allows regional specialisation of skin dependent on function. For example, the glabrous skin covering the palms of hands and soles of feet is relatively thick in structure, absent of hair follicles and sebaceous glands but has an increased amount of mechanoreceptors (Johansson and Vallbo, 1983; McGrath, Eady and Pope, 2004).

Adult human skin consists of a keratinised, stratified epithelial layer, with an underlying basement membrane, sitting above a relatively thick dermal layer (**Figure 1.1**). The zip-like interface between the epidermal rete ridges and dermal papillae gives the skin tensile strength and a robust barrier capacity. Below the dermis is the hypodermal layer containing mostly adipocytes. The hypodermis is conventionally described as cushioning, insulation and a functional energy reserve (Freinkel and Woodley, 2001). However, more recent research has shown its importance as an endocrine organ, releasing a range of factors which may be of particular significance during wound repair (Schmidt and Horsley, 2013; Musi and Guardado-Mendoza, 2014).

In addition to the aforementioned functions, the skin is also important for vitamin D production, protection from ultraviolet (UV) radiation and temperature regulation. Irregularities in cell types or proteins in the epidermis can lead to barrier defect phenotypes from the relatively mild such as atopic dermatitis (Agrawal and Woodfolk, 2014) to the severe such as epidermolysis bullosa (Varki *et al.*, 2007).

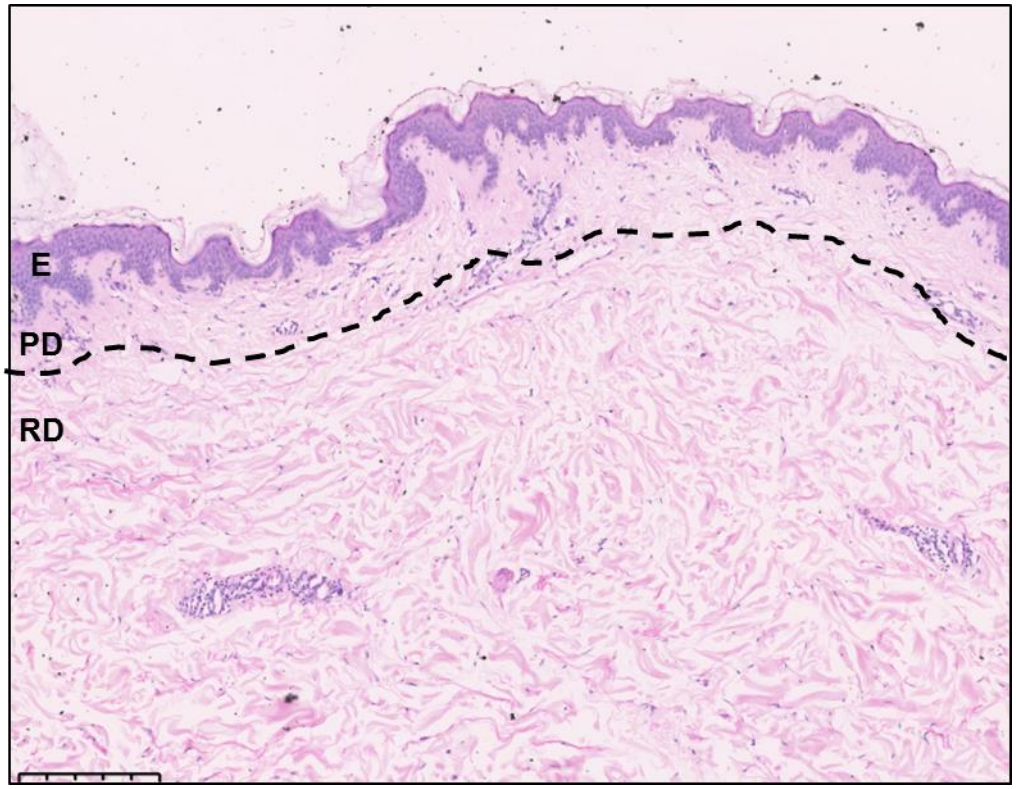



***Figure 1.1*** **- Normal skin histology showing microstructure and tissue architecture.** Formalin fixed, paraffin embedded normal skin section stained with haemotoxylin and eosin (H&E). Epidermis (**E**) is undulating with characteristical rete ridges. The dermis in two sections, the upper papillary (**PD**) and lower reticular dermis (**RD**) with typical loose, waved, basket weave pattern of collagen with cells scattered throughout the dermis. Scale bar represents 250µm.

### 1.1.2. Development of the skin

Mammalian skin is derived from two main compartments to form the top epithelial layer and the underlying dermis. The epithelial layer of the skin is known as the epidermis. The epidermis is derived from the same developmental niche as the nervous system, the neuroectoderm, early in mammalian development (Fuchs, 2007). From this preliminary single layered structure, cells proliferate and differentiate in response to environmental cues including Wnt and BMP signalling in order to progress to an epidermal fate (Figure 1.2) (Fuchs, 2007). It is interesting to note that these fate determining differentiation events seem to be cell specific (Fuchs, 2007) and may be due to intrinsic differences in these precursor cells which are not yet elucidated. Briefly, cells in this primitive layer that do not respond to Wnt signalling become the integral epidermal cells, whereas those that do respond to Wnt, and other signals including those from the underlying dermis, become appendages eventually resulting in hair follicle development (Figure 1.2) (Fuchs, 2007). This demonstrates the necessity of functional signalling between the dermal and epidermal compartment.

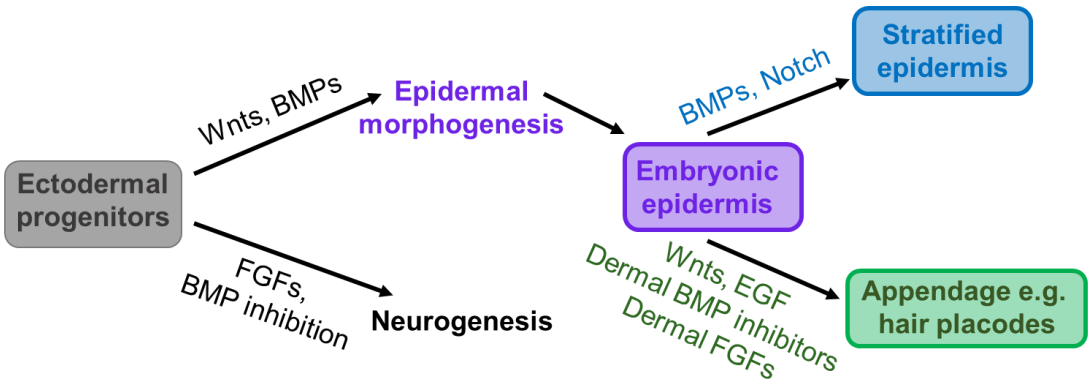



***Figure 1.2*: Schematic adapted from Fuchs, 2007 demonstrating the importance of signalling in epidermal development**. Embryonic epidermal cells are derived from the ectodermal progenitor cells that are responsive to Wnt and BMP signalling, these progenitor cells further specialise to become stratified epidermis, when responsive to BMP and Notch signalling, or appendage type epidermal cells such as those involved in hair follicle development.

Again, Wnt and β-catenin signalling is thought to be important in the development of the dermis, but much less is known about the cellular and molecular basis of dermal development. However, it is known that depending on the anatomical location, the dermis is derived from different locations, which may give rise to distinct functional characteristics later in mature skin. The ventral and flank dermis is derived from the lateral plate mesoderm, the head skin is derived from the neural crest ectoderm and the dorsal dermis is derived from the somatic dermatome (Olivera-Martinez, Thélu and Dhouailly, 2004). The transition from the somatic dermatome to dorsal dermis happens between gestation days 2 and 7 in chick (Olivera-Martinez, Thélu and Dhouailly, 2004), but still the molecular mechanisms for this transition are not well understood.

The skin continues to mature throughout gestation, with complete epidermal maturation by 34 weeks in humans (Oranges, Dini and Romanelli, 2015). Neonates tend to have a thinner epidermal layer with a weakened barrier function compared to adult skin, which continues to develop postpartum (Oranges, Dini and Romanelli, 2015).

### 1.1.3. The epidermis

The epidermis tends to be between 12.7μm and 16.6μm thick depending on anatomical location (Sandby-Møller, Poulsen and Wulf, 2003) and its structural bulk is made predominantly of keratinocytes. Stratified layers of keratinocytes are found in adult skin in five main layers, from the inner to outermost being: basal, spinous, granular, upper granular and cornified (Candi, Schmidt and Melino, 2005) The upper cornified layer of the epidermis, known as the stratum corneum, is formed by terminal differentiated, dead keratinocytes as well as various structural proteins, including involucrin and profillagrin, which give the skin further strength, resistance and control of water permeability (Candi, Schmidt and Melino, 2005) A small percentage of keratinocytes, resident in the basal layer of the epithelium retain multi-potency into adulthood and are integral in the maintenance of skin homeostasis but also the response to injury (Fernandes, Toma and Miller, 2008; Fuchs, 2008; Jones and Wagers, 2008). This population of cells is often described as a stem cell niche and has been heavily studied in the fields of developmental biology and oncology but also has a key role to play in wound healing as a pool for replacing lost cells (Watt, 1998).

Melanocytes are another important cell type in the epidermis, derived from the neural crest as melanoblasts which migrate, proliferate and differentiate to form mature melanocytes (Lin and Fisher, 2007). Melanocytes typically reside in the basal layer of the epidermis in close proximity to the dermis or the hair follicle bulbs. These cells are responsible for producing the pigment melanin, in granules known as melanosomes, which is important for UV protection as well as giving colour to skin and hair (Lin and Fisher, 2007). It is important to note that colour is determined by the number and size of melanosomes, rather than the number of melanocytes which is similar between individuals (Lin and Fisher, 2007). Mechanosensory cells called Merkel cells are also found in the epidermis and are important for the perception of touch and pressure (Maksimovic *et al.*, 2014). Debate has arisen as to the origin of these cells, but the most recent evidence suggests they are epidermally derived rather than derived from neural crest (Van Keymeulen *et al.*, 2009)

A range of immune cells are also present in the epidermis including specialised gamma delta T cells (known as dendritic epidermal T cells in mice, DETCs) and Langerhans cells (McGrath, Eady and Pope, 2004). Langerhans are abundant immune cells in the epidermis with a mesenchymal origin, making up 3 to 8% of epidermal cells (Katz, Tamaki and Sachs, 1979). As well as being a first line of defence in skin immunity (Merad, Ginhoux and Collin, 2008), these cells are highly important in skin immune homeostasis with the ability to both induce activation of regulatory T cells and effector memory T cells (Seneschal *et al.*, 2012). DETCs also contribute to the skin immune response (McGrath, Eady and Pope, 2004), as well as being early

and rapid responders to cutaneous injury by helping to drive the proliferative response of keratinocytes (Havran and Jameson, 2011).

### 1.1.4. The dermis and integral role of dermal fibroblasts

The dermal layer of the skin is a collagenous matrix of two stratifying layers, the reticular and the papillary dermis (**Figure 1.1**). The dermis is populated mainly by fibroblasts but also contains small populations of resident immune cells including a range of T cells subsets, dendritic cells and mast cells (Nestle *et al.*, 2009). The dermis holds blood vessels and a number of appendage structures including sebaceous glands and hair follicles (McGrath, Eady and Pope, 2004). Like the epidermis, there is also a dermal stem cell niche in the form of specialised dermal fibroblasts residing at the dermal papilla of hair follicles. These cells are thought to be important in the anagen phase of the hair cycle (Driskell *et al.*, 2013).

Fibroblasts are resident in many connective tissues including the dermis of the skin and are the main cell type of interest in this project. Fibroblasts were first described over 150 years ago and are characterised by their elongated, spindle-like appearance (Virchow, 1859). Despite existing as a stable non-proliferative population *in vivo*, dermal fibroblasts become highly proliferative in culture (Sorrell and Caplan, 2004). A vital role of all fibroblasts is to produce the extracellular matrix (ECM) that is vital for tissue structure, flexibility and integrity (Tracy, Minasian and Caterson, 2016). In the skin, fibroblasts tend to produce collagens I and III, however shortly after wounding they can efficiently produce immature ECM including fibrin and fibronectin to fill the wound bed (Tracy, Minasian and Caterson, 2016).

Dermal fibroblasts have been a strong focus in regenerative medicine due to their highly plastic and reprogrammable phenotype (Takahashi *et al.*, 2007; Nakagawa *et al.*, 2008). Fibroblasts are also capable of differentiation to myofibroblasts within the wound bed induced by signals including transforming growth factor beta 1 (TGF-β1) (Hinz, 2007). This plasticity will be further discussed in the context of wound repair later. It has also become clear in recent research that fibroblasts in the dermis are not a homogenous population but a collection of sub-populations that may have distinct functions in homeostasis and in the response to wounding (Driskell *et al.*, 2013). It has been identified in mouse dorsal skin that it is predominantly En1 positive, reticular dermal fibroblasts that contribute towards the fibrotic response seen in scar (Rinkevich *et al.*, 2014), although the equivalent population has not yet been identified in human.

## 1.2. Normal wound healing in the skin

### 1.2.1. An overview of the wound healing process

There are several major challenges to overcome after cutaneous wounding (**Figure 1.3**). Immediately after wounding, the critical problem is the physical gap and damage to the tissue, causing bleeding and a barrier breach for opportunistic pathogens. There is an immediate intervening reaction in the recruitment of the innate immune response and the formation of a blood clot, driven by a variety of cytokine and growth factor pathways (Martin, 1997). The activation of platelets shortly after wounding allows the formation of a haemostatic plug which is reinforced by the production of fibrin (Golebiewska and Poole, 2015). This fibrin clot gives a structure on which to re-build the epidermis from the wound edges. The rapid recruitment of neutrophils to the wound allows for clearing of invading pathogens and debris, as well as triggering downstream signalling that can promote other wound healing processes (Theilgaard-Monch *et al.*, 2004). Neutrophils are relatively short-lived in this environment and are phagocytosed by tissue resident macrophages, or monocyte derived macrophages from the circulation (Eming, Krieg and Davidson, 2007).

Meanwhile, the successive activation of keratinocytes to become migratory and proliferative, followed by the re-building of the dermal layer by fibroblast proliferation, ECM production and contracture, brings the wound edges together and reconstitutes the wound bed (Shaw and Martin, 2016).

The initial ECM tends to be rapidly produced and haphazardly arranged to create strength and protection, so this basic post-wound structure must be remodelled to regain normal skin function. Re-vascularisation by angiogenesis as well as the re-building of various appendages including sebaceous glands and hair follicles must occur. In many cases, especially in more extreme wounds, this remodelling is not entirely successful and will result in a scar with a loss of function (Xue and Jackson, 2015).

The plasticity of cells during repair is crucial at all stages for successful wound healing (Shaw and Martin, 2016). It is well-appreciated in the literature that wound repair is a tightly controlled and cross-regulating process, including vital paracrine signalling events between cell populations. Misregulation at any stage can therefore cause major issues in the repair process including failure to heal, the formation of aberrant scars, and development of cancer (Arnold *et al.*, 2015; Ge *et al.*, 2017).

For example, wound edge keratinocytes must be able to become migratory to ‘fill the gap’ in the epidermis, and fibroblasts must be able to become ‘activated’ to produce matrix, whilst being

able to return to a homeostatic phenotype following wound resolution. The cues for these differentiation events are not entirely characterised. It is thought in early stages of wound repair that the production of growth factors and cytokines may play a key role in initiating and driving these changes (Ploeger *et al.*, 2013), but the 'off switches' for many wound repair activities are less understood.

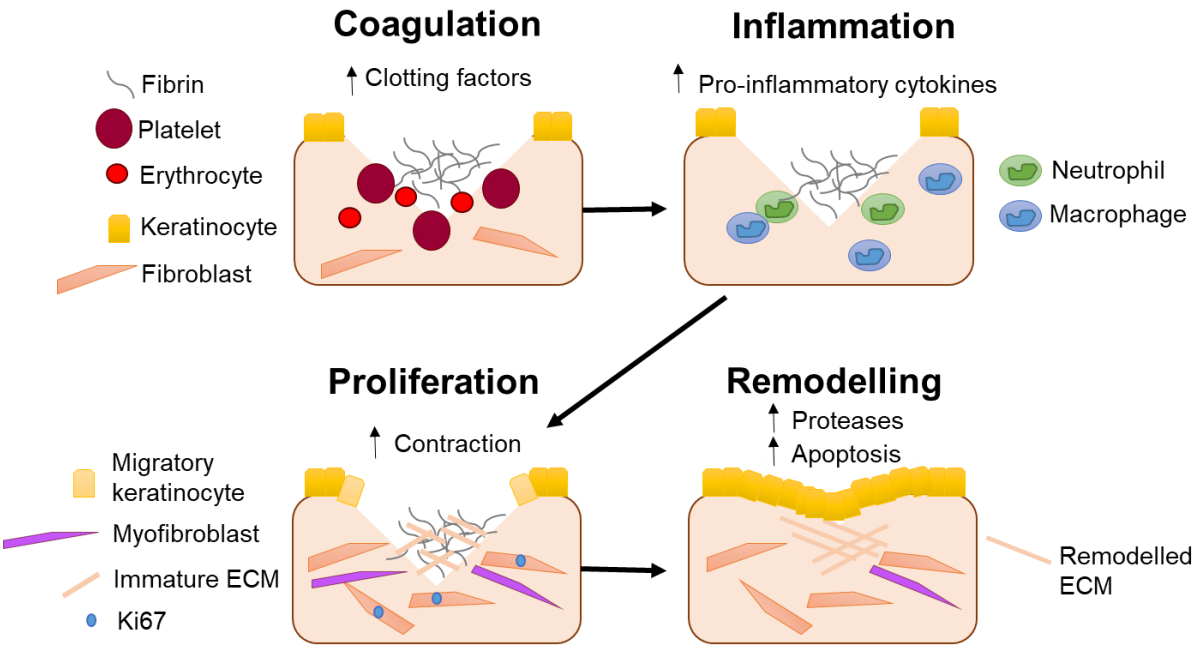



***Figure 1.3* - Schematic showing the main stages of cutaneous wound healing.** There are four main stages to cutaneous wound repair; although they are depicted as a consecutive, linear process these processes occur in tandem with overlapping phases of inflammation and proliferation for example.

### 1.2.2. Proliferation, migration and contraction in wound healing

Proliferation, migration and contraction of cells are all important processes that can occur concurrently in wound repair, and errors in these processes can cause disease. Firstly, proliferation of cells in wound repair is essential to replace cells that are lost during wounding; in the skin this tends to be mainly fibroblasts, and keratinocytes which also differentiate to reform the stratified epithelium that has been damaged (Bielefeld, Amini-Nik and Alman, 2013). Proliferation of fibroblasts in the skin is thought to be partially driven by β-catenin, thought to be independent of Wnt signalling (Bielefeld *et al.*, 2011). Interestingly TGF-β1, which is known as another strong inducer of fibroblast proliferation, has an anti-proliferative effect on keratinocytes (Pietenpol *et al.*, 1990). Proliferation can also be driven through cell-cell interactions, such as Langerhans cells and keratinocytes (Morhenn, 1988) and through paracrine signalling between keratinocytes and fibroblasts (Werner, Krieg and Smola, 2007).

Migration of cells, especially wound edge keratinocytes is vital for successful re-epithelisation (Shaw and Martin, 2016). Cells often collectively migrate in a sheet in a fashion reminiscent to embryonic morphogenesis (Jiang *et al.*, 2013) and the advancing cells also deposit immature matrix in order to form a substrate for this movement (Shaw and Martin, 2016).

Fibroblast contraction is also important in order to pull the wound edges together and is driven by TGF-β1 and mechanical tension in the environment (Hinz *et al.*, 2001). There is a subset of dermal fibroblasts that can differentiate into specialised, highly-contractile myofibroblasts which are characterised as being alpha-smooth muscle actin (α-SMA) positive (Hinz, 2007). However, there is also strong evidence that there are important contractile α-SMA negative fibroblasts, as shown in α-SMA-null mice which still elicit significant contraction upon wounding, which may be reliant on beta and/or gamma cytoplasmic actin (Ibrahim *et al.*, 2015).

### 1.2.3. Inflammation in wound healing

Inflammation is highly influential in wound repair; as well as neutrophils and macrophages, other inflammatory cells including adaptive immune cells such as T cell subsets also influx to the wound and may regulate processes including matrix remodelling (Havran and Jameson, 2011). The importance of some other immune cells, such as mast cells, is less clear (Willenborg *et al.*, 2014).

Several studies have attempted to unravel the importance of inflammation in wound repair using immune-deficient models such as the PU.1 null mouse. PU.1 is the master transcription factor for the myeloid lineage, therefore PU.1 null mice lack a range of immune cells including macrophages, dendritic cells and T cells (Martin *et al.*, 2003). Despite typically dying shortly post-birth from opportunistic infections, they have been shown to generate scar-free wounds upon injury (Martin *et al.*, 2003). This study supports the idea that there is a necessity of balance in the immune response after wounding, preventing pathogen invasion yet avoiding excessive scar formation through typical inflammation-driven responses.

Based on this evidence, it is logical to suggest that application of anti-inflammatory or immunosuppressive compounds could be used beneficially to promote scar-free healing, especially through modulation of macrophage activity. Macrophages can be polarised along a spectrum of pro-inflammatory (M1) to anti-inflammatory (M2), and there is a great deal of debate as to the functional importance of these cells to wound repair (Eming, Krieg and Davidson, 2007). It has recently been shown that M2-like macrophages can indirectly contribute to scarring through the production of insoluble scar-type cross-linked collagen by fibroblasts through IL-4 signalling (Knipper *et al.*, 2015). It has been demonstrated that the relevance of macrophages varies temporally, with the depletion of macrophages early in repair showing detrimental effects in mice but having much less effect later in repair (Lucas *et al.*, 2010).

### 1.2.4. Matrix remodelling in wound healing and a role for MMPs

After the initial filling of the connective tissue gap with immature matrix, ECM is remodelled over time, driven chiefly through protease activity, to return the tissue as close to the original state as possible (Lu *et al.*, 2011). A fine balance must be struck in regards to protease levels as an increase in proteolytic activity may cause damage to local tissue or non-healing wounds such as seen in diabetic foot ulcers (Mast and Schultz, 1996; McCarty and Percival, 2013), whereas a lack of proteolytic activity may result in excessive ECM deposition leading to unresolved scar tissue (Ghosh and Vaughan, 2012).

There are many proteases that have roles in the remodelling of ECM including adamlysins (ADAM), cathepsins, hyaluronidases, heparanases, cysteine proteases and plasminogens (Roycik, Fang and Sang, 2009). However, the most well characterised of these proteases are the matrix metalloproteinases (MMPs) (Roycik, Fang and Sang, 2009; Rohani and Parks, 2015). A range of cells in the skin can contribute to MMP production (**Table 1.1**). MMPs are best recognised for their ability to remodel matrix (Rohani and Parks, 2015). MMPs can cleave a range of substrates in tissue, many of which may not yet be defined but include those that are typically found in repaired skin matrix such as collagen I, III and fibronectin (Yates, Hebda and Wells, 2012).

MMPs can also cleave a range of other proteins including growth factors and cell surface receptors, causing a plethora of downstream cellular consequences (**Figure 1.4**). Cleaved bioactive peptides, known as matrikines, generated from degradation of ECM proteins are also capable of regulating cellular processes by receptor engagement. For example, proline-glycine-proline fragments have been shown to trigger chemotaxis of neutrophils (Wells, Gaggar and Blalock, 2015). Also, the regulation of growth factor activation, such as TGF-β1, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) by MMPs is further evidence for their importance and the need for regulation of their activity (Sternlicht and Werb, 2009) and may explain why MMP1 is able to mobilise the migration of wound edge keratinocytes in the early stage of wound repair (Martin, 1997; Rohani and Parks, 2015).

It has indeed been shown that MMPs are elevated in both benign (Denys *et al.*, 2004) and malignant tumours (Kessenbrock, Plaks and Werb, 2010) driving growth, and metastasis in the case of cancer. For example, it is known that MMPs 2, 9 and 14 are important for driving angiogenesis in the tumour microenvironment (Kessenbrock, Plaks and Werb, 2010) particularly through the release and activation of VEGF (Bergers *et al.*, 2000). Moreover, it is likely that MMPs also have further functions that are yet to be defined (de Veer *et al.*, 2014),

***Table 1.1* - Summary of likely sources of MMP in skin/scar tissue.**

| | Enzyme type | Potential sources in the skin |
|---|---|---|
| MMP-1 | Collagenase | Fibroblasts, keratinocytes, melanocytes* |
| MMP-2 | Gelatinase | Fibroblasts, keratinocytes |
| MMP-3 | Stromelysin | Fibroblasts, keratinocytes, melanocytes* |
| MMP-7 | Matrilysin | Sweat gland epithelium, fibroblasts |
| MMP-8 | Collagenase | Mostly neutrophils, fibroblasts |
| MMP-9 | Gelatinase | Neutrophils, fibroblasts, keratinocytes, eosinophils, melanocytes* |
| MMP-10 | Stromelysin | Fibroblasts, keratinocytes |
| MMP-11 | Stromelysin | Fibroblasts, melanocytes* |
| MMP-12 | Elastase | Macrophages, fibroblasts |
| MMP-13 | Collagenase | Macrophages, fibroblasts, *chondrocytes* |
| MMP-14 (MT1-MMP) | Membrane associated | Fibroblasts, keratinocytes, macrophages |

*= melanocytes in melanoma (healthy melanocytes do not make MMP-11), *chondrocyte differentiation may be apparent in keloid fibroblasts*

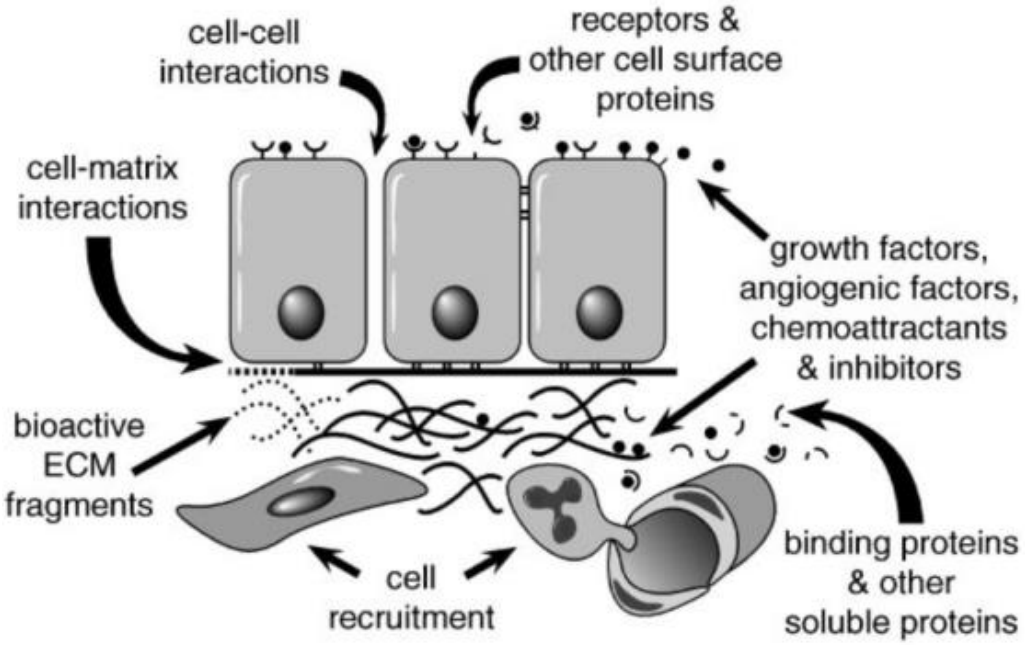



***Figure 1.4* - Schematic summarising non-structural changes induced by MMPs that may have functional relevance in pathology** (from Sternlicht & Werb 2009).

## 1.3. Scarring and fibrosis

### 1.3.1. Spectrum of wound repair success, from regeneration to scarring

The perfect response to a wound would be the complete regeneration to a normal resting state. In early stages of foetal development there is an inherent ability to regenerate after a wounding event leading to scar-free healing (Leung, Crombleholme and Keswani, 2012). Indeed, in some organisms, such as the planarian flatworm or those in the Caudata order, this ability is retained into adulthood (Tanaka and Reddien, 2011). These organisms are therefore intensively studied as models of regeneration and can provide useful information in the context of wound healing. Axolotyl, a salamander species, is able to regenerate entire limbs following amputation, requiring the accurate development of multiple tissue types (Tanaka and Reddien, 2011). This process clearly requires tight regulation in order to retain regenerative ability upon wounding whilst not spontaneously growing unnecessary tissue. The regulation of these processes are heavily studied by the regenerative medicine field in the hope that this will allow the development of tools to regenerate tissue and organs in humans (Roy and Gatien, 2008; Seifert *et al.*, 2012).

Importantly, even within mammals there is differential ability to regenerate between different organs. The liver is extraordinarily regenerative upon injury, with the ability to regrow after two thirds of its mass is removed. However, the central nervous system is renowned for being very poor in repairing wounds, with intense research focus on improving the prognosis of those with spinal cord injuries who typically suffer from excessive scarring and loss of normal organ function resulting in paralysis (Fitch and Silver, 2012). The skin is also less successful in healing wounds, especially large and full thickness wounds like burns which tend to result in hypertrophic scars (van den Broek *et al.*, 2014).

Scar tissue is characteristically fibrotic and is associated with some loss of architecture and function in the tissue (Xue and Jackson, 2015). For example, in the skin there is an altered structure and content of matrix, with the loss of normal flexibility and elasticity, there is also a failure to redevelop functional appendages including hair follicles and sebaceous glands (Bayat, McGrouther and Ferguson, 2003). The reason for the generation of scars is not well understood but it is proposed that it is an evolutionary by-product of a rapid wound repair process that is prioritised to protect from a pathogenic environment (Allen and Wynn, 2011). It has also been suggested that scar tissue formation can be a protective mechanism against further insults to that area (Gauglitz *et al.*, 2011).

### 1.3.2. Common mechanisms in scarring and fibrosis

Fibrosis can occur as a result of injury, or other causes of inflammation, including infection (Wynn, 2013). Fibroblasts are the driving cell type in this pathology as they are responsible for the production of matrix (Kendall, Feghali-Bostwick and Rauch, 2014). Persistent activation of myofibroblasts can also drive fibrosis and can be promoted by paracrine signals from immune cells, autocrine signals from other myofibroblasts or even through interaction of pathogens via fibroblast toll-like receptors (TLRs) (Wynn, Yugandhar and Clark, 2013). Increasingly, epigenetic mechanisms are being linked to fibrosis, including influences on myofibroblast activation (Mann and Mann, 2013).

Chronic inflammation often correlates with fibrosis with a presumed causal relationship both through innate and adaptive arms of immunity (Wynn, Yugandhar and Clark, 2013). Persistence of innate immune cells, such as neutrophils and then macrophages, can cause tissue damage. High neutrophil counts have been associated with severity of disease in idiopathic pulmonary fibrosis (IPF) (Obayashi *et al.*, 1997) and the release of extracellular chromatin structures by neutrophils, called neutrophil extracellular traps, causes the myofibroblast differentiation of lung fibroblasts (Chrysanthopoulou *et al.*, 2014). Depletion of neutrophils in an infection driven pulmonary fibrosis model ameliorated the disease (Puerta-Arias *et al.*, 2016).

There is even stronger evidence for the influence of both pro-inflammatory "M1" and pro-fibrotic "M2" skewed macrophages on fibrosis (Wynn and Vannella, 2016). It appears that both subtypes can drive fibrosis, either through the production of TGF-β1, as noted in IPF (Murray *et al.*, 2011) or indirectly through the activation of Th2 responses and IL-13 secretion (Kaviratne *et al.*, 2004) (**Figure 1.5**). Depletion of macrophages in fibrosis models have shown their importance in both pulmonary and liver fibrosis with a decrease in disease severity (Duffield *et al.*, 2005; Gibbons *et al.*, 2011).

Coagulation has also been linked to fibroses in liver and lung, with the neutralisation of coagulation factors II, V, VII, IX and XI preventing fibrosis in animal models (Scotton *et al.*, 2009; Mercer and Chambers, 2013). It also appears that dysregulation of vasculature can influence fibrosis. Interestingly, it has been noted in systemic sclerosis (SSc) that a loss of normal vasculature often occurs prior to the onset of fibrosis (Varga and Abraham, 2007), whereas in many fibrotic eye conditions, there is uncontrolled proliferation of unstable blood vessels that then leak inflammatory products, further fuelling fibrosis (Friedlander, 2007) and an increased angiogenic stage has been seen in hypertrophic versus normal skin that may promote scar growth (Van Der Veer *et al.*, 2011).

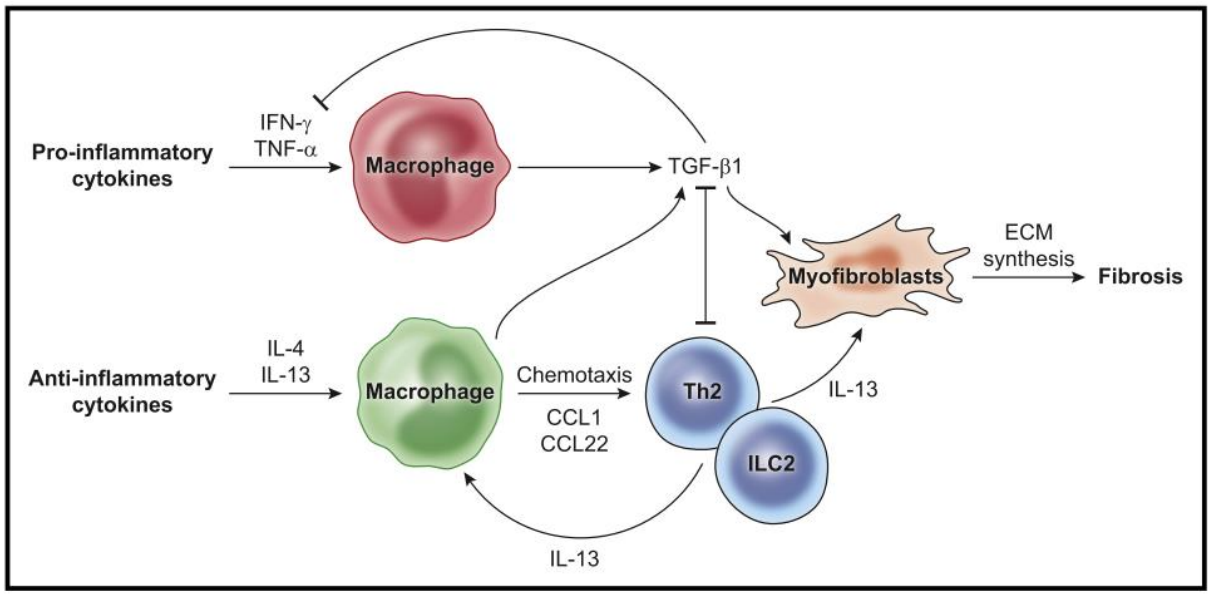



***Figure 1.5* - Schematic demonstrating macrophage influence on fibrosis** from (Wynn and Vannella, 2016) where the red macrophage represents "M1" and the green macrophage represents "M2"

Adaptive immunity can also influence fibrosis through the actions of polarised CD4 T cells (Wynn, 2004). Helper T cells can generate two alternative responses: Th1, which is characterised as an interferon type, phagocyte dependent inflammation; or Th2, which is characterised as an IL-4 driven response that is phagocyte independent (Romagnani, 2000). It appears that the balance of Th1 and Th2 responses is critical, with a more Th2 heavy response tending to exacerbate fibrosis (Wynn, 2004; Allen and Wynn, 2011). This was first demonstrated in an IL-12 based vaccination against schistosome mediated fibrosis which successfully primed a Th1 heavy response to attenuate fibrosis (Wynn *et al.*, 1995).

B cells have also been implicated in fibrosis, although this is more controversial and has only been shown convincingly in liver fibrosis models so far (Novobrantseva *et al.*, 2005) with variable results in SSc (Hasegawa *et al.*, 2006).

### 1.3.3. Role of growth factors and cytokines in scarring and fibrosis

Since fibrosis is driven by the pathological behaviour of fibroblasts, it is important to understand factors that are capable of activating fibroblasts and influencing their behaviour (Wynn, Yugandhar and Clark, 2013). There are a number of growth factors and cytokines, especially interleukins, reported in the literature to be associated with fibrosis (Borthwick, Wynn and Fisher, 2013; Wynn, Yugandhar and Clark, 2013), the most prevalent of which will be described in the following sub-sections. The inclusion of the following growth factors and cytokines is by no means inclusive of every factor that may be involved in fibrosis, or specifically keloid pathology, but represent some of the most well characterised and reported.

#### 1.3.3.1. TGF-β

Transforming growth factor beta (TGF-β) is one particularly well characterised factor that is implicated in the fibroses of many organs (Meng, Nikolic-Paterson and Lan, 2016). The family member TGF-β1 has been shown to be an important driver of inflammation during cutaneous wound healing (Wang *et al*. 2006) and indeed mice lacking SMAD3, a key mediator of TGF-β signalling, have accelerated wound healing associated with a decrease in inflammation during repair (Ashcroft *et al.*, 1999). However, TGF-β1 is frequently reported as 'out-of-control' in the formation of abnormal, excessive scars (Beanes *et al.*, 2003) and could also play a role in driving a pro-fibrotic M2 macrophage response that can further exacerbate fibrosis (Wynn and Vannella, 2016). A landmark study in 1995 demonstrated the TGF-β1 and TGF-β2 isoforms are pro-fibrotic in a wound healing context, whereas TGF-β3 is anti-fibrotic (Shah, Foreman and Ferguson, 1995).

Signalling through the SMAD complex is thought to drive a variety of pathological cell behaviours including the differentiation and persistance of myofibroblasts which produce excessive collagen and can be persistently contractile (**Figure 1.6**). Recently it has been shown TGF-β1 may drive a scar phenotype through upregulation of microRNA 21 (miR21) (Liu *et al.*, 2016). Equally, activation of downstream SMADs can drive a plethora of cell behaviours through transcription, including the production of profibrotic miRs and long non-coding RNAs (lncRNAs) (Meng, Nikolic-Paterson and Lan, 2016). Indeed, many miRs have been shown to regulate different stages of TGF-β/Smad signalling (Meng, Nikolic-Paterson and Lan, 2016). SMAD7 is a negative regulator of pro-fibrotic TGF-β signalling, and its disruption or lowered can also worsen fibrosis (Zhu, Chen and Chen, 2011). It has additionally been shown that there

is cross-talk between TGF-β signalling and other important fibrosis pathways including MAPK, p38, JNK and ERK pathways (Meng, Nikolic-Paterson and Lan, 2016).

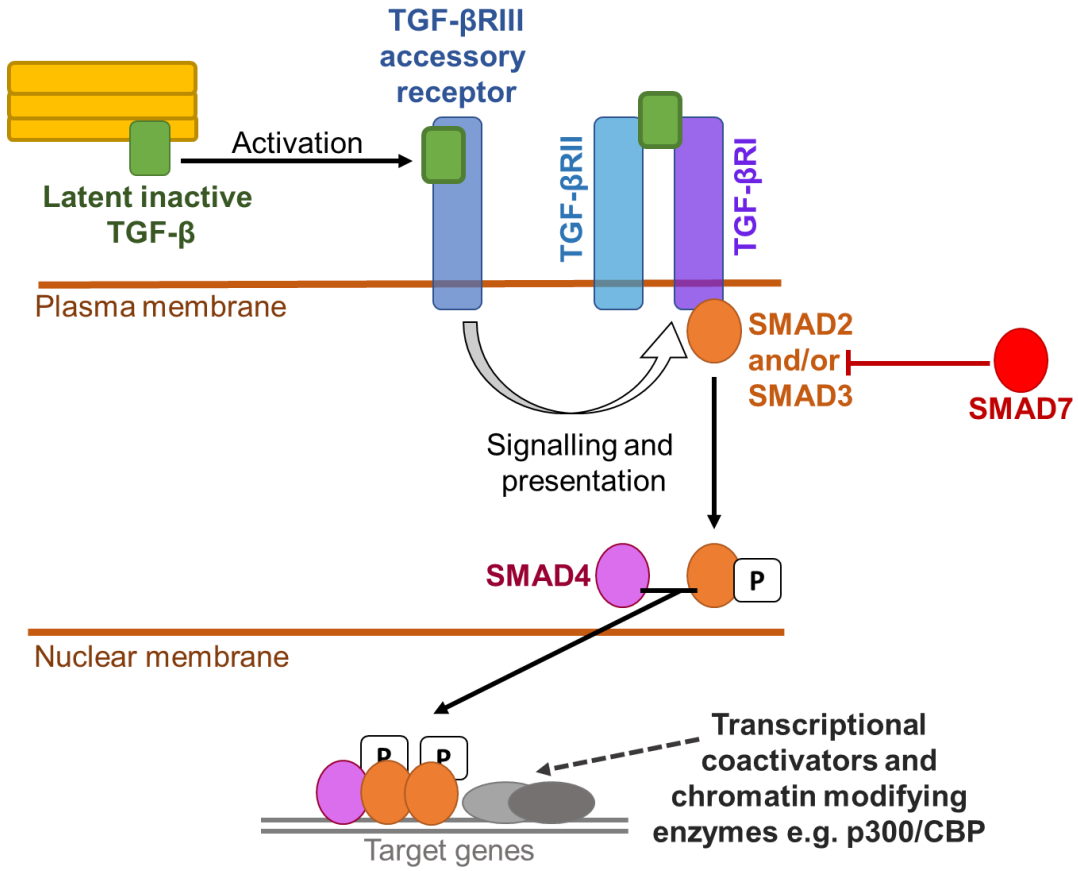



***Figure 1.6* - Schematic showing profibrotic TGF-β signalling ( adapted from Varga & Abraham).** TGF-β1, often cleaved from a latent form bound to ECM, engages with TGFβ receptors to activate SMAD signalling, causing phosphorylation and nuclear localisation of SMAD proteins to drive target gene transcription. SMAD7 works as a negative regulator in this system to inhibit downstream signalling.

#### 1.3.3.2. PDGF-BB

The platelet derived growth factor (PDGF) family contains five isoforms, PDGF-AA, -AB, -BB, -CC and -DD which act by the two receptors PDGF-α and -β (Fredriksson, Li and Eriksson, 2004). Upregulation of both PDGF ligands and associated receptors has been reported in fibrosis (Bonner, 2004). PDGF has been described as the most potent driver of stellate cell proliferation in liver fibrosis, although the isoform was not stated (Pinzani *et al.*, 1989). Different PDGF isoforms are postulated to be more important in the fibrotic process in certain organs, such as PDGF-BB in skin fibrosis (Bonner, 2004). Much less is known about PDGF-CC and -DD isoforms but they have been implicated in renal fibrosis (Eitner *et al.*, 2003; Ostendorf *et al.*, 2003). Interestingly, recombinant human PDGF-BB has been used clinically (known as becaplermin) to improve healing in diabetic foot ulcers (Fang and Galiano, 2008)

PDGF can trigger ERK and PI3K/Akt signalling, but is also thought to overlap with TGF-β1 to drive fibrosis (Bonner, 2004). PDGF stimulation of hepatic stellate cells increases the profibrotic transcription of connective tissue growth factor (CTGF) which can be blocked with the neutralisation of TGF-β1 (Paradis *et al.*, 1999). Additionally, TGF-β1 stimulation of SSc fibroblasts in culture can cause upregulation of PDGFRα (Yamakage *et al.*, 1992).

#### 1.3.3.3. Th2 cytokines – IL-4/IL-13

Cytokines that are associated with Th2 response, namely IL-4 and IL-13, are thought to drive fibrosis (Wynn, 2004). The strongest evidence for an elevation of these cytokines comes from SSc patients where IL-4 and IL-13 are increased in patient serum compared to healthy controls (Hasegawa *et al.*, 1997). IL-4 and IL-13 share the co-receptor IL-4rα and both signal primarily through the signal transducer and activator of transcription factor 6 (Stat6) pathway (Oh, Geba and Molfino, 2010). Both cytokines have been shown to drive myofibroblast differentiation (Hashimoto *et al.*, 2001) and cause increased collagen production in hepatic stellate cells (Sugimoto *et al.*, 2005). IL-4 in particular has highly potent effects on dermal fibroblasts, with IL-4 stimulation of cells from normal skin or SSc patients causing over twice the amount of collagen production than an equivalent dose of TGF-β1 (Fertin *et al.*, 1991).

These cytokines have many important effects on matrix production, aside from collagen. IL-4, and particularly IL-13, have been shown to induce the production of the matrix protein periostin (Takayama *et al.*, 2006; Izuhara *et al.*, 2016) which has been shown to promote fibrosis in many organs including the heart (Zhao *et al.*, 2014), lungs (Naik *et al.*, 2012) and skin (Yamaguchi,

2014). Stimulation of fibroblasts with IL-4 also stimulates the production of fibronectin, an important component of scar matrix (Babu *et al*. 1989). In addition, it has been shown in mouse injuries that IL-4 triggers downstream signalling in M2-like macrophages that subsequently causes the induction of the enzyme lysyl hydroxylase-2 from dermal fibroblasts. This enzyme catalyses the production of cross linked, irregularly sized collagen bundles that are more resistant to degradation and therefore pro-fibrotic (Knipper *et al.*, 2015).

In addition to an increase in full length IL-4, a differentially spliced isoform called IL-4$\delta$2 has also been identified in the blood of SSc patients (Sakkas *et al.*, 1999). The functional effect of IL-4$\delta$2 is not entirely clear but there is evidence to show that it does induce fibrotic behaviour of fibroblasts through hyper-proliferation and collagen production (Atamas and White, 1999; Atamas *et al.*, 1999).

IL-13 also appears to be a highly pro-fibrotic cytokine and can activate M2 macrophages to drive the development of fibrosis (Wynn and Barron, 2010). Overexpression of IL-13 in the lung showed development of fibrosis (Zhu *et al.*, 1999) and conversely IL-13 inhibition attenuated models of fibrosis in the liver and lung (Chiaramonte *et al.*, 1999; Murray *et al.*, 2014). It has additionally been demonstrated that IL-13 driven Th2 responses can drive fibrosis in the absence of TGF-β1 (Kaviratne *et al*. 2004). IL-33, an IL-1 family member, has also been shown to be correlative with fibrotic severity in disease including SSc (Yanaba *et al.*, 2011) and is a well-recognised driver of Th2 responses through ST2 signalling (Murakami-Satsutani *et al.*, 2014)

#### 1.3.3.4. IL-17A

IL-17A, also referred to as IL-17, is generally regarded as a pro-inflammatory cytokine produced by a CD4+ T cell subset known as Th17s, as well other innate immune cells including neutrophils (Jin and Dong, 2013). IL-17 has been found at increased levels in fibrotic liver, and IL-17R deficient mice show improved outcomes in a carbon-tetrachloride induced model of liver fibrosis which may be driven by a reduced neutrophil influx (Tan *et al.*, 2013). This study also showed that IL-17 stimulation of murine hepatic stellate cells induces production of key fibrotic markers including IL-6, α-SMA and collagen (Tan *et al.*, 2013)

Conversely, whilst IL-17 expression is increased, both locally in the skin, and systemically in SSc patients, the level of the relevant receptor, IL-17R, is decreased in SSc fibroblasts (Nakashima *et al.*, 2012). The authors suggests that, as these cells have a limited capacity to respond, IL-17 ligand is in fact acting in an anti-fibrotic manner in this system (Nakashima *et*

*al.*, 2012). The role of IL-17 ligand in fibrosis is therefore unclear and may be related to the cell type affected.

#### 1.3.3.5. IL-6

IL-6 is often referred to as a pro-inflammatory cytokine, however its function *in vivo* is distinctly pleiotropic (Scheller *et al.*, 2011). The importance of IL-6 in wound healing has been shown with both IL-6 and IL-6r knockout mice showing wound healing defects (McFarland-Mancini *et al.*, 2010). However, persistent high levels of IL-6 is associated with fibrosis in a range of organs (Fielding *et al.*, 2014).

To respond to IL-6, cells must express both the co-receptor GP130, which is ubiquitously expressed across cell types, and the IL-6r whose expression is more stringent (Heinrich *et al.*, 1998). However, soluble IL-6r (sIL-6r) can also be produced by the cleavage of membrane bound receptor, or the expression of a spliced isoform lacking the transmembrane domain (Heinrich *et al.*, 1998). The presence of soluble receptor therefore allows a greater spectrum of cells to respond to IL-6 in the environment. It has been shown that an inflammatory environment, which is also likely to have elevated levels of IL-6, can promote cleavage of IL-6r, especially from infiltrating neutrophils (Rose-John, 2012), causing a greater response from the local cellular environment.

There is debate in the literature whether fibroblasts are capable of expressing IL-6r, or whether they may rely on sIL-6r in the environment in order to respond to IL-6. However, it has been shown that stimulating fibroblast cultures with IL-6 alone can promote proliferation and collagen production (Ghazizadeh *et al.*, 2007) suggesting that fibroblasts do express IL6r. Downstream of IL-6r, IL-6 effects are largely though to be mediated by Jak/Stat3 signalling (Heinrich *et al.*, 1998), which is purported to be highly pro-fibrotic (Zhang *et al.*, 2005; Knight, Mutsaers and Prêle, 2011). However, IL-6 is known to be capable of triggering other pathways including Akt and Erk driven signalling which may explain its highly pleiotropic effects (**Figure 1-7**).

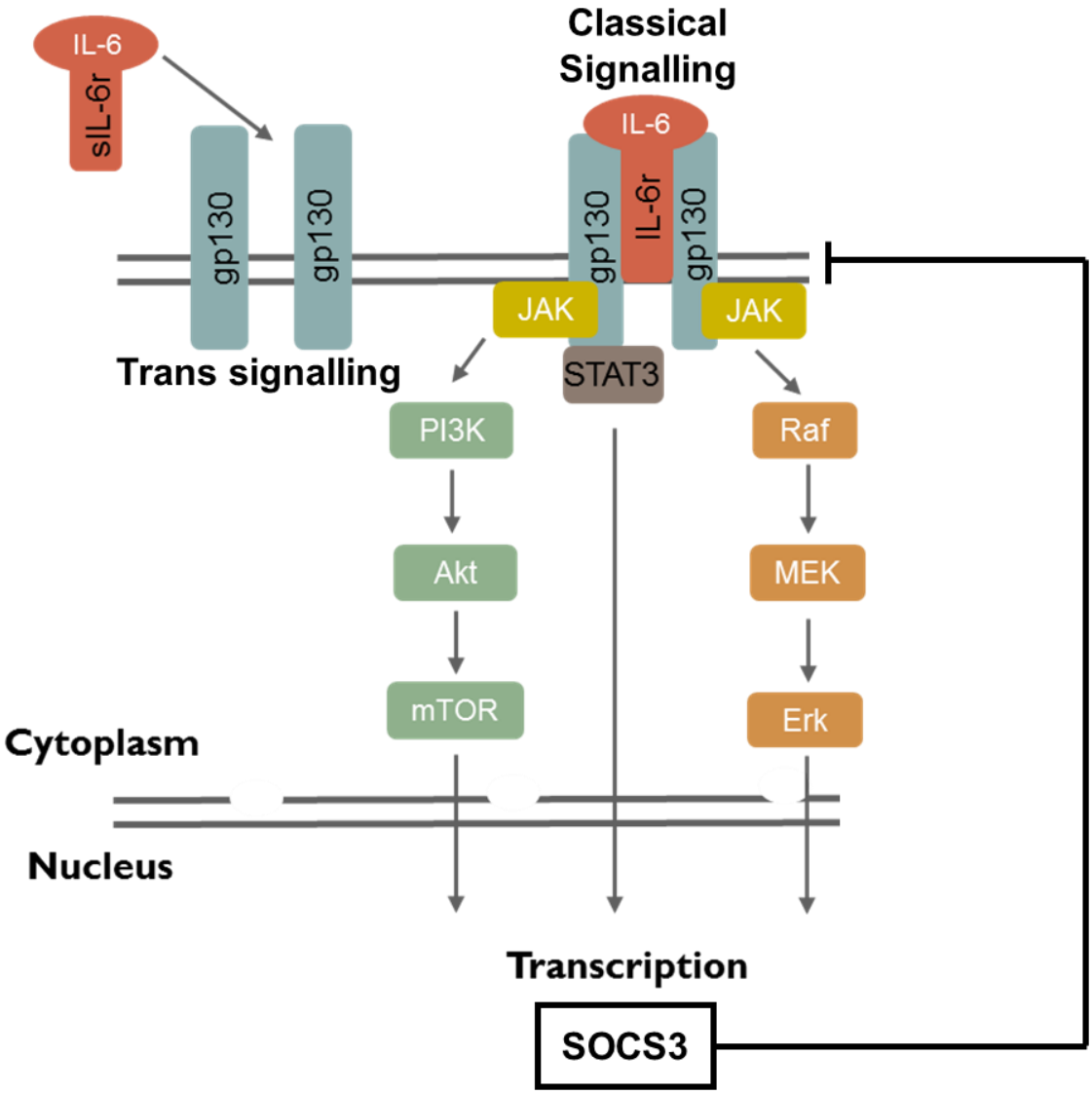



***Figure 1-7* - Schematic demonstrating possible pathway activation downstream of IL-6.** IL-6 ligand requires the presence of GP130 and IL-6r (membrane bound or soluble form, sIL-6r) to signal. Signalling using sIL-6r is known as trans signalling, whereas signalling using the membrane bound IL-6r is known as classical signalling. After receptor engagement, dimerization and phosphorylation of Jaks can cause phosphorylation and downstream signalling through Stat3, PI3K/Akt and Raf/MEK/Erk to result in nuclear localisation of transcription factors and transcription can occur. A plethora of genes may be transcribed following this signalling event, including SOCS3 which can negatively regulate signalling by binding to elements including GP130 and Jak2.

## 1.4. Keloid scarring

### 1.4.1. Scarring in the skin

Erroneous control of the cutaneous wound repair process can cause a spectrum of abnormalities including the non-healing ulcers seen in diabetic patients to the formation of fibrotic scars (Martin, 1997). It is predicted that every year 100 million patients in the developed world alone form scars. The majority of human scars are normotrophic, forming after relatively minor wounds to the skin, and causing a very minor cosmetic issue (Bayat, McGrouther and Ferguson, 2003; Verhaegen *et al.*, 2009). These scars are generally flat and pale and are not associated with contracture or itching/pain (Bayat, McGrouther and Ferguson, 2003).

Histologically, normal scars are distinct to the adjacent normal skin with a relatively clear border in the two tissue types (**Figure 1.8**). The scarred area has a flattened epidermis (**Figure 1.8**), with loss of the usual rete ridge and dermal papillae structures (Rook, 1996), but the overall epidermis is a similar thickness to the normal skin (Limandjaja *et al.*, 2017). The dermal area of scar has compact, smooth collagen (**Figure 1.8**) instead of the classical basket weave pattern of collagen bundles (Bayat, McGrouther and Ferguson, 2003).

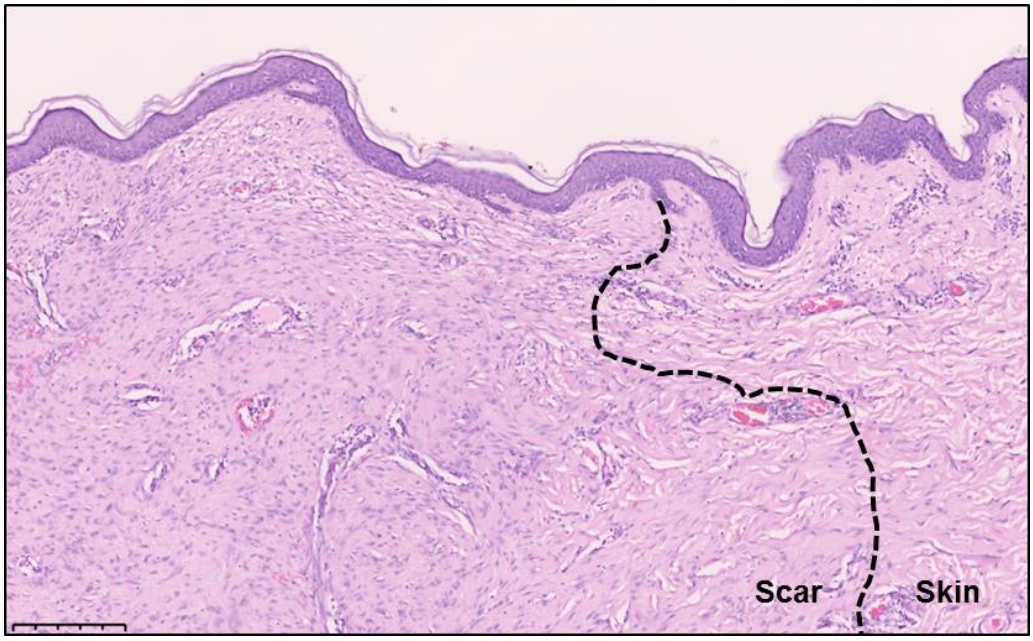



***Figure 1.8*** **- Histological features of normal scar/normal skin.** Formalin fixed, paraffin embedded normal skin/normal scar section stained with H&E. Dashed line denotes approximate normal skin/scar border. Typical scar pathology with loss of normal undulating epidermis and a dense matrix. Normal skin shows typical basket weave collagen in dermis and usual rete ridge structure in epidermis. Scale bar represents 250μm.

Although normotrophic scars are most common after wounding, scars can occur with a range of severity (**Figure 1.9**). Hypertrophic scars are a prominent scar that are confined within the original wound region and typically partially resolve with time (Muir, 1990). They often occur after full thickness wounds such as those obtained from surgical procedures or burns (Bayat, McGrouther and Ferguson, 2003).

Keloid scars are a more severe form of scarring and can expand well outside of the wound margin, often causing a tumour-like mass (Bayat, McGrouther and Ferguson, 2003). These scars do not resolve with time and will frequently continue growing for many years post injury (Muir, 1990). Susceptible individuals can generate a keloid scar in response to relatively minor wounds to the skin including insect bites and acne (Gauglitz *et al.*, 2011). Keloid scarring is a distinct fibrotic condition, with excessive inflammation and deposition of matrix proteins leading to dysfunction of the tissue. As in other fibroses, this atypical matrix-dense environment can functionally contribute to atypical cellular responses (Wells, 2008; Rhee, 2009). The clinical distinction between hypertrophic and keloid scars is often difficult so scars are sometimes instead diagnosed as an intermediate raised scar (Sidgwick, McGeorge and Bayat, 2015).

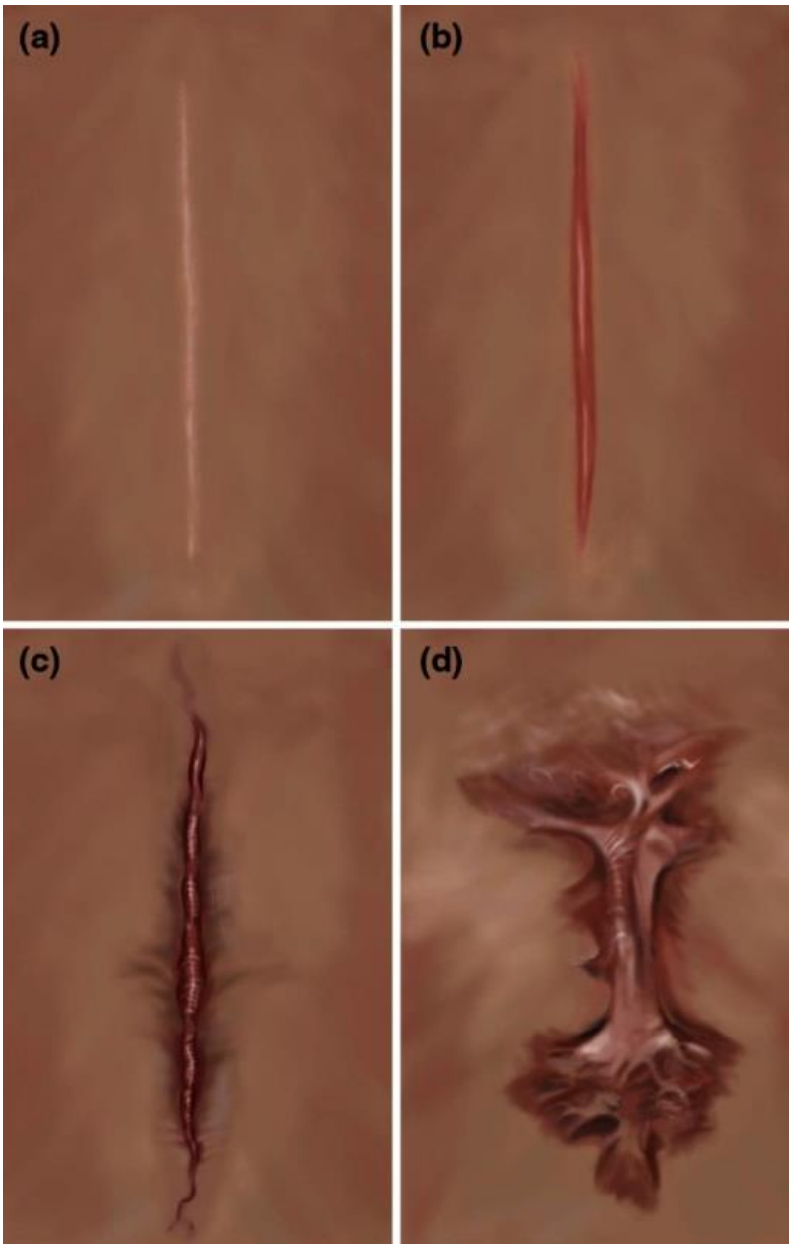



***Figure 1.9* - Representation of scar types (from Sidwick *et al* 2015).** a) A typical scar resulting from a common injury, known as a normotrophic scar, b) a more prominent scar often resulting from burns or surgical injury known as a hypertrophic scar, c) intermediate raised scar and d) a severe scar growing outside the wound margins known as a keloid scar.

### 1.4.1. Epidemiology and genetic risk of keloid scarring

Keloid scarring can affect any individual but is commonly associated with darker skin. In some populations, occurrence can be up to 16% (Rockwell, Cohen and Ehrlich, 1989). Keloids are reported to be more common in females with a possible hormonal influence (Gauglitz *et al.*, 2011). Evidence for a hormonal link is also supported by the observation of increased keloid risk during adolescence and pregnancy and decreased keloid risk post-menopause (Moustafa and Abdel-Fattah, 1975; Kim *et al.*, 2013).

In general, patients are not reported to present with any other clinical issues and show normal wound healing in all other organs. There have been a very small number of co-morbidity reports of keloids which although infrequent could indicate a genetic link to disease. One such condition is Rubinstein-Taybi syndrome (Van De Kar *et al.*, 2014), which results from mutations in histone acetyltransferases CREB binding protein (CBP) and/or EP300 where around 57% of patients have been found to generate keloid scars (Stevens, Pouncey and Knowles, 2011).

No clear genetic link has yet been established despite substantial anecdotal evidence of multiple family members suffering from the condition (Bayat *et al*. 2004). In general, there is a lack of large worldwide consortium genetics studies to inform on causative polymorphisms, however the pattern of keloid inheritance has been described to be consistent with an autosomal dominant disorder with incomplete penetrance (Marneros *et al.*, 2001). TGFB1 or corresponding receptor polymorphisms were originally described in 1991 to be a likely culprit (Peltonen *et al.*, 1991), however this has been since dismissed (Bayat *et al*. 2004) . A more recent study by Nakashima *et al* found 4 single nucleotide polymorphisms (SNPs) in NEDD4 and FOXL2 and in two other unknown regions (Nakashima *et al.*, 2010). The limitation of this study is the restriction of analysis to only Japanese keloid sufferers (824 patients and 3205 race matched controls) and it is likely that these polymorphisms may be race specific. The most statistically robust polymorphisms identified are in the human leukocyte antigen (HLA) domains which are likely to be important in controlling the immune response during wound repair, in particular HLA-DRB1*15 which has been identified in both Caucasian and Chinese population studies (Brown *et al*. 2008; Lu *et al*. 2010).

### 1.4.2. Clinical presentation and treatment of keloid scars

Keloid derives from the Greek word for ‘crab claw’ which illustrates the typical irregular, sprawling appearance of the scar as it grows outside of the original wound margin (**Figure 1.9**). Patients often suffer not only from aesthetic concerns but also pain, pruritus and loss of mobility if located over a joint (Davidson *et al.*, 2009). Keloid formation has a stronger tendency to occur in distinct anatomical locations including the back of the head, face, neck, chest and shoulder regions (**Figure 1.10**) (Alster and Tanzi, 2003).

Treatment for keloid scars is currently limited and variable in success. As a first point of call, clinicians will often attempt steroid injection with corticosteroids such as triamcinolone acetonide (Andrews *et al.*, 2016). It is estimated that around half of treated patients will be categorised as steroid non-responders and the explanation for this is not yet elucidated (Ud-Din *et al.*, 2013). Aside from pharmacological success, there are also issues with patient compliance due to the pain associated with the procedure. Surgical removal is seen as a last-resort option due the risk of recurrence which is estimated to be at least 40% and up to 100% in some studies (Rockwell, Cohen and Ehrlich, 1989). Surgical approaches for removal differ, however generally a healthy tissue border will not be taken. Treatments are often combined, such as surgical excision followed by corticosteroid injection, to reduce recurrence risk (Andrews *et al.*, 2016). Other less invasive options include compression or silicone bandages, as well as laser or radiotherapy (Gauglitz *et al.*, 2011) .

The issue of drug delivery to keloid scars, as in solid oncogenic tumours (Sriraman, Aryasomayajula and Torchilin, 2014), is important to consider when developing new therapeutics. Discussions with dermatologists and surgeons have suggested a limited capacity for success in topical treatments due to the dense matrix composition of the scar, and even intralesional injection of steroids has presented issues in fully infiltrating the keloid tissue. Approaches including hyperthermia and ultrasound (Polat *et al.*, 2011) are currently being researched in order to tackle the issue of permeation of tissue. Many alternative treatments are in research however an epigenetic approach may be more successful in reprogramming affected cells to promote successful repair, offering a more persistent cellular effect.

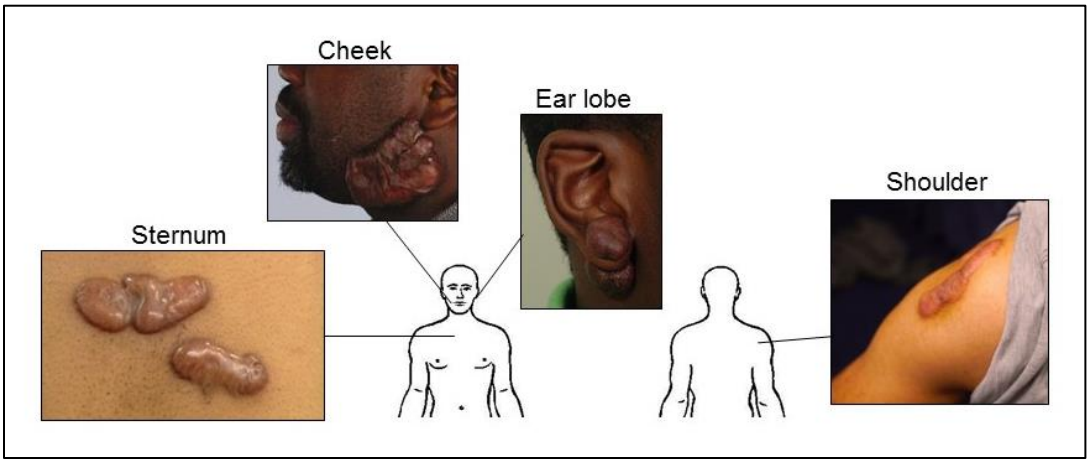



***Figure 1.10* - Photographs showing examples of keloids in the commonly occurring locations.** Collated from Google Images.

### 1.4.3. Histopathology of keloid scars

Histological analysis of keloid scars further reveals their abnormal composition (**Figure 1.11**). Keloid scars often show a thickened epidermal layer (Chua *et al.*, 2011) and although not clear from histological staining, an unusual basement membrane structure is also formed with a disruption of the normal permeability of this layer (Hellstrom *et al.*, 2014). Keloid dermis is highly cellular and rich with excessive and abnormal ECM with large bundles of hyalinised collagen structures and distinct clusters of immune cells (**Figure 1.11**). The distinction between the reticular and papillary dermis seen in normal skin is also lost in keloids. Notably, toluidine blue staining gives the appearance of cartilage in some keloid samples suggesting a possible mis-differentiation of the tissue (Naitoh *et al.*, 2005).

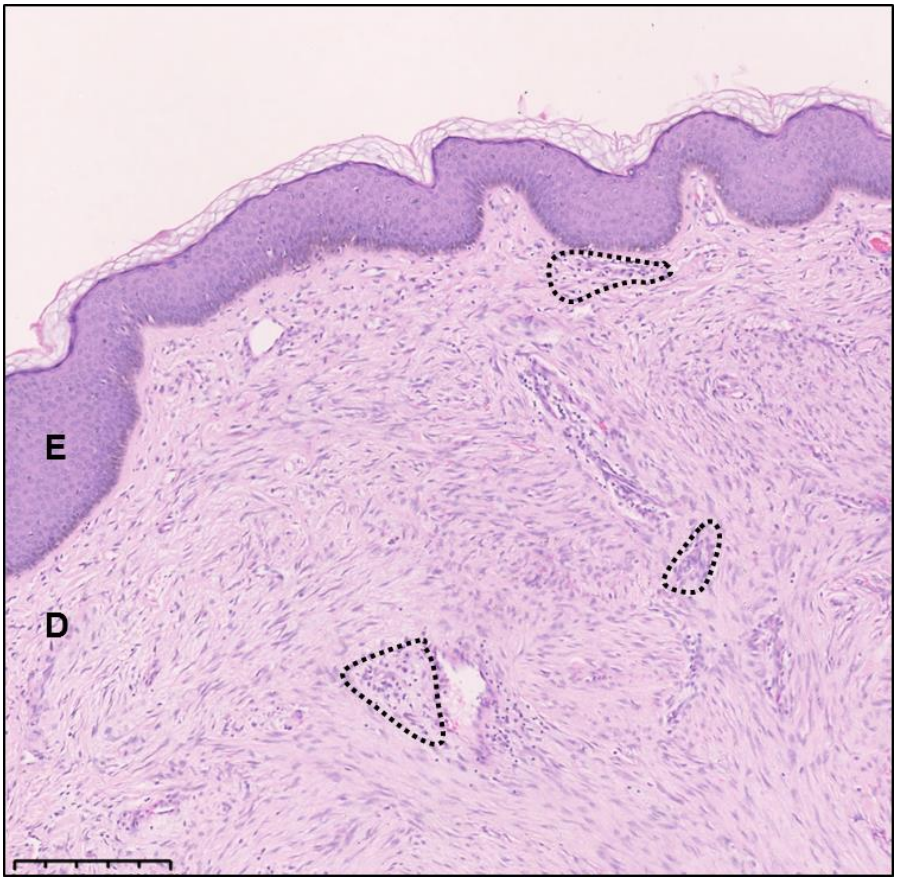



***Figure 1.11*** **- Histological features of keloid scar.** Formalin fixed, paraffin embedded keloid section stained with H&E. Typical keloid pathology with thickened epidermis (E) (around 100-200µm thick) with loss of normal rete ridge structure and a highly cellular dermis (D) with dense matrix characterised by swirling, hyalinised collagen bundles and distinct clusters of cells likely to be immune cells (examples highlighted with dotted lines). Scale bar represents 250µm.

### 1.4.4. Cellular makeup of keloid scars

#### 1.4.4.1. Keloid epidermal cells

Despite an obviously abnormal epidermal layer in keloid scars, it is not well understood how keratinocytes in this layer are different to that of normal skin. Keloid keratinocytes do not appear to be more proliferative (Hahn *et al.*, 2013; Limandjaja *et al.*, 2017). Instead these cells may be abnormally differentiated with expression of the early differentiation marker involucrin throughout the epidermis rather than restriction to the granular epidermal layer as is seen in normal skin and a disorganised, immature stratum corneum (Limandjaja *et al.*, 2017).

Transcriptomic analysis of keloid keratinocytes has shown decreased expression of transcription factors, including family members of the frizzled transcription factor family, and cell adhesion components that are required for normal keratinocyte biology including intermediate filament genes synemin (SYNM) and VIM, and desmosome junction components JUP and PKP1. Whereas keloid keratinocytes showed elevated expression of genes associated with wound healing, migration and vascular development such as keratin 7 (KRT7), and lysyl oxidase 2 (LOXL2) which has been implicated in ECM cross linking, tumour progression and cellular invasion (Hahn *et al.*, 2013). All of these transcriptomic changes suggest these cells have a changed phenotype indicating a chronic stage of erroneous healing.

The importance of cross-talk between the epidermis and dermis has been shown in the regulation of hair follicle development (Fu and Hsu, 2013) and it has already been proposed that the epidermis has a key role to play in dermal fibrosis in the context of hypertrophic scarring (Bellemare *et al.*, 2005). There is evidence that keloid keratinocytes can influence fibroblasts in a pathological way, as normal fibroblasts cultured with keloid keratinocytes show increased keloidal collagen production which was not seen in culture with normal keratinocytes (Lim *et al.*, 2002). A paracrine factor(s) released by keloid keratinocytes-fibroblast co-cultures could also enhance fibroblast collagen gel contraction (Mukhopadhyay *et al.*, 2005). Keloid keratinocytes have additionally shown a fibroblast-like morphology in hypoxic conditions, suggesting enhanced epithelial-mesenchymal transition (EMT) potential compared to normal skin (Ma *et al.*, 2015).

No clear evidence has been found to show that melanocytes can drive keloid scar formation, but it has been hypothesised based on the higher incidence in darker skin (Gao *et al.*, 2013) and a single research paper shows a downregulation of the α-melanocyte-stimulating hormone and its associated receptor, melanocortin-1 in keloid scar tissue (Luo *et al.*, 2013).

### 1.4.4.2. Keloid immune cells

Despite evidence that keloid scars are often very inflamed, and this inflammation is thought to drive keloid pathogenesis (Ogawa, 2017) the immune cell population in keloid tissue is not well defined. As seen in histological sections of keloids (**Figure 1.11**), immune cells appear to reside in distinct clusters in the papillary dermal layer and these clusters have been described in the literature as KALTs (keloid associated lymphoid tissue) (Bagabir *et al*. 2012a; Grant *et al*. 2016), similar to the MALTS (mucosal associated lymphoid tissue) seen in the gut during intestinal inflammation (Lochner *et al.*, 2011). This would suggest that these populations are long-lived and stable, providing paracrine factors that may cause, exacerbate, or prolong keloid establishment and growth.

An immunohistochemical (IHC) approach by the Bayat group showed increases in almost all immune cell sub-types examined, including T-cells, B-cells, mast cells and macrophages, although no apparent difference in Langherhans cells (Bagabir *et al*. 2012a). However, some of the markers used may not offer enough specificity, for example, major histocompatibility complex II (MHC-II) can be expressed by a range of activated cells such as CD4 T-cells (personal communications, Esperanza Perucha). Attempts by our lab to better characterise the keloid immune cell population by FACS on fresh tissue have been technically challenging but has indicated a tipped balance towards M2 type macrophages (data not shown). A skew towards a M2 phenotype is likely to propagate the keloid pathology, with the production of M2 associated factors including TNF-α, and IL-6, which is a particular focus for this project and is likely to induce pathological fibroblast behaviour (Xue, McCauley and Zhang, 2000; Tosa *et al.*, 2005; Ghazizadeh *et al.*, 2007)

### 1.4.4.3. Keloid fibroblasts

Fibroblasts are the predominant cell type in the dermal layer of the skin, where keloid pathology is particularly prominent (Smith *et al.*, 2008), as well as being a key effector cell in this disease. During the 1980s and 90s, a number of papers were published describing differing characteristics in keloid and normal dermal fibroblasts. These include a greater proliferative capability (Harper, 1989; Calderon, Lawrence and Banes, 1996), and production of pro-fibrotic markers including excessive ECM (Babu, Diegelmann and Oliver, 1989; Bettinger *et al.*, 1996). Since then, the availability of larger scale analysis through '-omics' studies has allowed for deeper analysis of these differences on a protein and genetic level. A microarray study comparing cultured normal scar and keloid scar fibroblasts indicated differential regulation of

around 500 of 38,000 genes analysed, showing increased insulin-like growth factor binding proteins, and decreased Wnt inhibitors and IL-1 inducible genes, indicating a pro-fibrotic phenotype (Smith *et al.*, 2008). However, in general, although transcriptomic data has shown some common trends, such as inflammation and fibrosis associated pathways, gene lists tend to be inconsistent due to variability between study set-ups (Smith *et al.*, 2008; Suarez *et al.*, 2015; Jumper *et al.*, 2017). There is still a need to further this research in order to control studies appropriately and correlate them to relevant phenotypic behaviours.

Despite a lack of *in vitro* evidence for heightened keloid fibroblast proliferation, these cells have shown increased thymidine incorporation in a scratch "wound" assay in comparison to normal skin fibroblasts, suggesting these cells respond differently to damage cues (Calderon, Lawrence and Banes, 1996). Above just being more 'activated', it has been postulated that these cells may in fact be mis-differentiated away from a skin phenotype. There is evidence that keloid fibroblasts are de-differentiated, expressing a number of stem-like markers (Zhang *et al.*, 2009; Grant *et al.*, 2016), or differentiated to a more osteogenic or chondrogenic lineage (Naitoh *et al.*, 2005; Fuentes-Duculan *et al.*, 2016). Interestingly, proteomic analysis from our lab alongside evidence of an increase in the chondrogenic transcription factor Sox9 (data not shown) provides more evidence that this could be true, pushing highly plastic dermal fibroblasts (Shaw and Martin, 2016) towards a chondrocyte-like fate producing abnormal ECM not usually seen in normal skin or normal scar.

The source of the fibroblast population found in keloid scars is debated in the literature. They may originate from an existing fibroblast population that has become unnaturally proliferative and activated (Driskell *et al.*, 2013), potentially because of the influence of the immune cell infiltrates in keloid scars (Ogawa, 2017). However, there are alternative hypotheses in the literature (**Figure 1.12**). Circulating fibrocytes may be recruited from the bloodstream (Abe *et al.*, 2001), or transdifferentiated fibroblasts can be induced from epithelial cells through EMT (Yan *et al.*, 2010). It has been shown that EMT can occur during cutaneous wound healing and marked by vimentin/E-cadherin double positive cells and high levels of Snail1 and MMP9; this may be linked to an upregulation of the transcription factor Foxn1 (Gawronska-Kozak *et al.*, 2016).

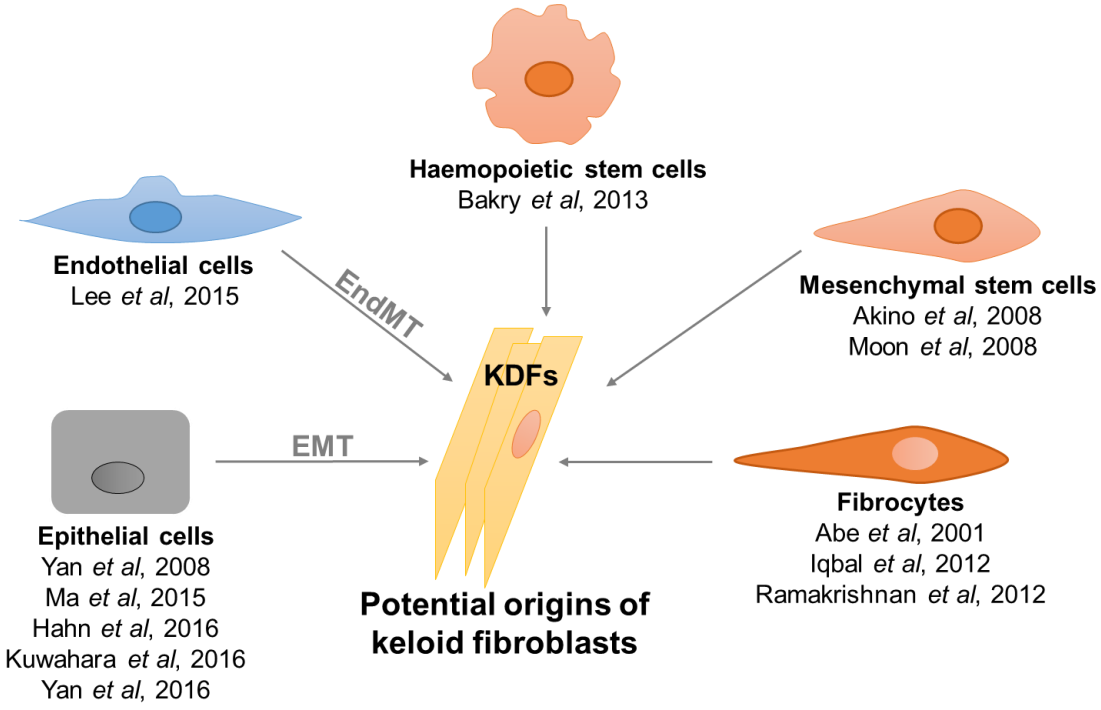



***Figure 1.12*** **- Schematic representing possible alternative origins of keloid fibroblasts.**

### 1.4.5. Current literature hypotheses on keloid aetiology

The mechanisms behind keloid scar formation are currently unknown. However, it is an accepted paradigm that inflammation is a key driver in this pathology, due to the persistence of immune cells as previously described. Prolonged presence of immune cells can induce a pro-inflammatory environment in the wound which can also promote scarring through activation and proliferation of fibroblasts (Wynn, 2004; White and Mantovani, 2013; Fielding *et al.*, 2014). Keloid scars in particular are associated with a high level of inflammation which can persist many years after the wounding event (Ogawa, 2017). Elevated levels of growth factors and cytokines have been shown to be present within keloids, with indication of raised cytokine levels also seen in the circulation of these patients (McCauley *et al.*, 1992). Various studies have examined the secretory immune environment in keloids, and two factors that are consistently reported as increased are TGF-β1 and IL-6 (Xue, McCauley and Zhang, 2000; Tosa *et al.*, 2005; Ghazizadeh *et al.*, 2007; Uitto, 2007).

The growth factor TGF-β1 is a proposed driver of keloid scarring due its recognised role in fibrosis (Varga, Rosenbloom and Jimenez, 1987; Leask and Abraham, 2004) with a critical increase in the ratio of TGF-β1 to TGF-β3 in the keloid environment that could tip the balance towards a fibrotic phenotype (Bock *et al.*, 2005; Jagadeesan and Bayat, 2007). There may also be differences in TGF-β receptor expression on keloid fibroblasts that cause diverse signalling events in response to stimulation from different TGF-β isoforms (Bock *et al.*, 2005). Given the classic pro-fibrotic association with downstream IL-6 signalling through Jak-Stat3 signalling (Heinrich *et al.*, 1998), this is now been a focus for discussion in regards to therapeutic strategies (Ghazizadeh *et al.*, 2007; Uitto, 2007) and will be further explored within this project.

It is also thought that there is a diminished yet prolonged remodelling phase in keloid aetiology with a possible persistence of myofibroblasts causing tension within the wound (Chipev *et al.*, 2000). This persistent contractility, may cause pain, and may also cause sustained inflammation. There is also suggestions that there may be an increase in protease activity at the leading edge of keloids, which may allow for their continuous outgrowth (Sadick *et al.*, 2008; Thielitz *et al.*, 2008).

A variety of historical, less-supported theories for keloid formation include an auto-immune response to sebum after wounding (Yagi, Dafalla and Osman, 1964), elevated levels of histamine (Russell, Russell and Trupin, 1977) and defective induction of senescence in keloid fibroblasts (Blažić and Brajac, 2006).

Evidence for the possible mis-differentiation of keloid fibroblasts, along with difficulties in finding definitive causative genetic loci for the disease despite familial inheritance being

reported, could suggest that there is an epigenetic level of regulation that may be responsible for keloid formation and this requires further investigation.

## 1.5. Epigenetic control in wound healing and fibrosis

Epigenetics is the term to describe the regulatory control of gene transcription and therefore cell behaviour and fate (Helin and Dhanak, 2013). This regulation occurs at multiple levels and allows strict control of cells in a dynamic environment.

Dermal fibroblasts are eminently associated with a highly plastic phenotype (Osonoi *et al.*, 2011), hence their application in regeneration research and therapy (Wong, McGrath and Navsaria, 2007). These cells may have more flexibility in their behaviour and fate supported by a more flexible epigenetic signature (Plikus *et al.*, 2015), which is particularly important in terms of their response to cutaneous wounding (Shaw and Martin, 2016).

Epigenetic regulation may occur at a variety of levels, including directly on histones. For example, after wounding the leading edge keratinocytes show flexibility in their behaviour, marked by epigenetically "un-silencing" by removal of polycomb-deposited histone marks that normally control keratinocyte identity and behaviour (Shaw and Martin, 2009). However, deletion of EZH2, an important component of the polycomb complex, in cardiac progenitors caused hypertrophy and fibrosis (Delgado-Olguín *et al.*, 2012) and its overexpression in IPF fibroblasts caused myofibroblast differentiation (Xiao *et al.*, 2016), highlighting the need for tight, cell-specific control of its expression to regulate homeostatic cell behaviour.

Increased flexibility of the epigenome through "open" chromatin can facilitate deleterious effects in response to insults (Yang, Tian and Brasier, 2017), including wounding (Neary, Watson and Baugh, 2015), regardless of genetic sequence. Various epigenetic signatures have been linked to fibrosis such as hypomethylation, which has been associated with fibrosis in the liver (Komatsu *et al.*, 2012), lung (Huang *et al.*, 2014), eye (McDonnell, O'Brien and Wallace, 2014) and more recently in keloid scarring (Jones *et al.*, 2015). Myofibroblasts, which are thought to be a key driver of tissue fibrosis are also thought to be epigenetically regulated (Mann and Mann, 2013).

MiRs are well discussed in recent literature in the context of wound healing (Banerjee and Sen, 2015; Fahs *et al.*, 2015) and are in fact vital during normal skin development (Yi *et al.*, 2006). MiRs can both positively and negatively regulate every stage of wound repair (Fahs *et al.*, 2015) with a fine balance to be struck. Therefore, mis-expression of specific miRs can have drastic impacts on cell behaviour and a knock on effect in disease. For example, 29 miRs were found to

be differentially expressed between scratched wounded diabetic corneal cells compared to healthy controls, and knockout of miR-146a or miR-424 in particular decreased wound closure (Funari *et al.*, 2013). miRs have also been heavily implicated in fibrotic disease, and are of particular interest due to their often very tissue-specific effect.

## 1.6. Epigenetic changes in keloid scarring

The epigenetic influence in keloid aetiology is still not fully explored but some convincing evidence for epigenetic changes has been elucidated. Keloid fibroblasts have been shown to have differential DNA methylation and histone acetylation patterns compared to normal scar fibroblasts (Russell *et al.*, 2010). Altered DNA methylation was also seen in keloid tissue, with 685 CpG sites differentially methylated compared to normal skin (Jones *et al.*, 2015). In addition, elevated levels of histone deacetylase 2 (HDAC2) has been shown in normal and keloid scars, which is thought to be driven by TGF-β1 and high cellular density (Fitzgerald O′Connor *et al.*, no date).

MiRs have also been identified as differentially expressed in keloid scar tissue, and specifically in fibroblasts (He *et al.*, 2017). Despite some inconsistencies between studies, some were common across multiple studies including a decrease in miR-199a, a decrease in miR-200c and most convincingly (seen across three independent studies) an increase in miR-21 (He *et al.*, 2017). MiR-21 is thought to increase proliferation and decrease apoptosis through destabilisation of phosphatase and tension homolog (PTEN) with an consequential increase in downstream Akt signalling (Liu *et al.*, 2014). Upregulation of certain miRNAs in response to sex specific hormones including oestrogen (Klinge, 2009) may give some explanation of the higher keloid occurrence in females and an increased risk of formation during pregnancy (Kim *et al.*, 2013).

Differential expression of over 1200 lncRNAs has also been seen in microarray analysis of three paired earlobe and normal skin samples (Guo *et al.*, 2016). Notably, a lncRNA called H19, which is usually associated with oncogenic tumours, was increased in keloids compared to normal skin and scar tissue, and siRNA targeting of H19 decreased proliferation, mTOR and VEGF signalling (Zhang *et al.*, 2016).

All the epigenetic changes observed so far are likely to have functional relevance, and offer a new angle for therapeutic targeting in order to “normalise” diseased cell behaviour in keloid scars.

## 1.7 BET protein family

### 1.7.1. Bromodomain containing proteins

Bromodomains (BRDs) are 110kDa regions within a number of proteins, including BET proteins (**Figure 1.13**). BRDs are structured as of a four-helix bundle forming a hydrophobic pocket in which acetylated lysine residues can bind (Zeng and Zhou, 2002). There are thought to be over 36,000 lysines that can be acetylated in human cells, as described by PhosphoSitePlus®. Although BRD interaction with acetylated lysine is commonly described on histones, functioning as epigenetic regulators, these proteins have also been shown to bind to non-histone associated lysine residues (Sanchez and Zhou, 2009).

Many proteins contain BRDs, including histone acetyltransferases (HATs) such as CBP and EP300, transcription factors, and transcriptional silencers including transcription intermediary factors (TRIMs) (Muller, Filippakopoulos and Knapp, 2011). Proteins containing BRDs often contain other functional domains that drive the specificity of their interactions with chromatin and other proteins. For example BRD4 contains two BRDs but also contains an N-terminal domain region which interacts with positive transcription elongation factor b (P-TEFb) to recruit RNA polymerase II (RNA pol II) (Yang *et al.*, 2005) and is thought to increase binding stringency through specific binding to genomic DNA (Hnilicová *et al.*, 2013)

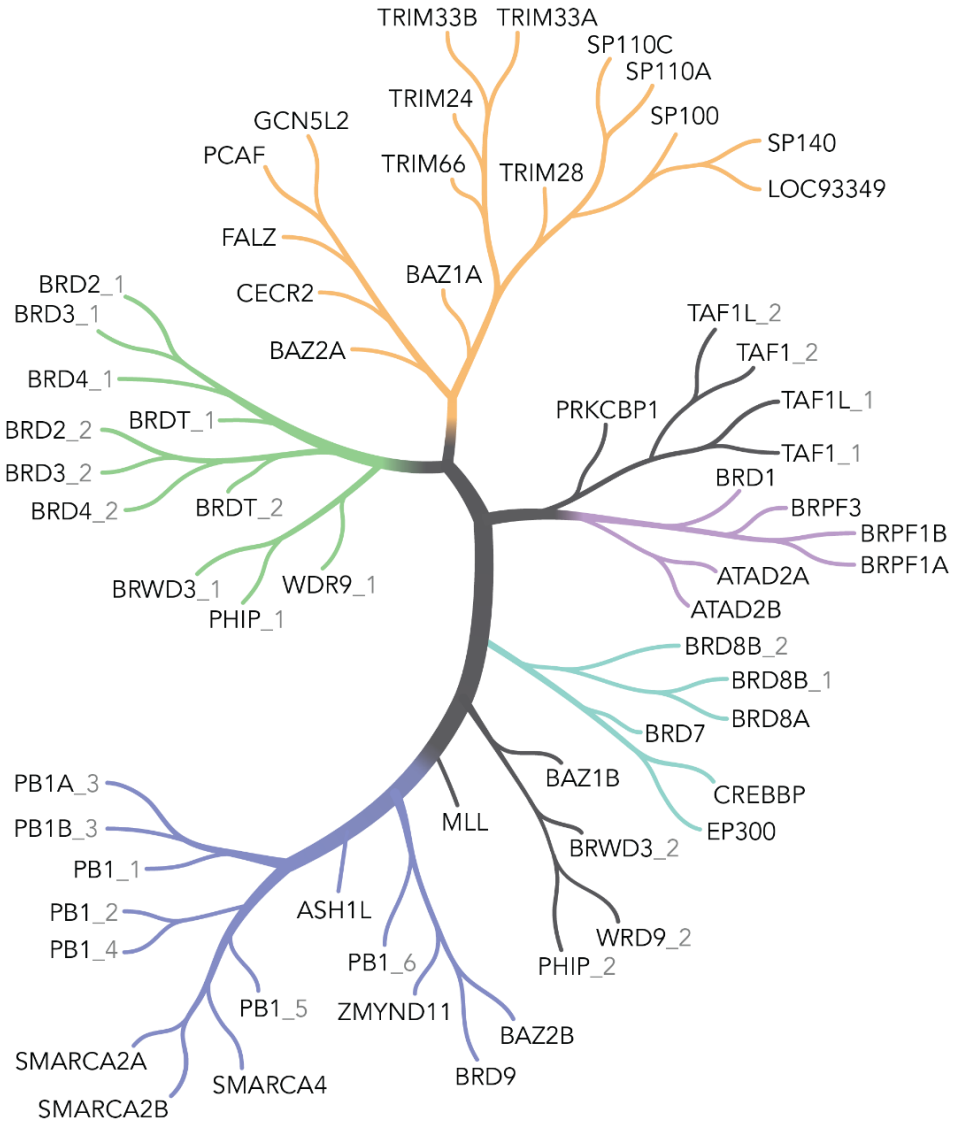



***Figure 1.13*** **- Network of bromodomain (BRD) containing proteins.** Image from: https://www.horizondiscovery.com/cell-lines/all-products/explore-by-your-research-area/bromodomain-containing-proteins showing relatedness of BRD domains.

### 1.7.2. BET members- BRD2, BRD3, BRD4, BRDT

The BET protein family contains four human members: BRD2, BRD3, BRD4, and BRDT which is uniquely expressed in the testes. All four members are characterised by their overall structure with two BRDs and an N- extra terminal (NET) domain (**Figure 1.14**). BRD2, previously called RING3, was first reported as a nuclear kinase, with homology to a developmental gene in *Drosophila* called female sterile homeotic (*Fsh*) (Denis and Green, 1996). BETs appear to be highly conserved, with proteins homologous to human BRD4 seen in mouse, *Xenopus*, and zebrafish as well as *Drosophila* (Toyama *et al.*, 2008).


***Figure 1.14* - Schematic of typical BET protein structure.** BRD = bromodomain, NLS = nuclear localisation signal and NET = N extra terminal domain.

BETs are thought to be ubiquitously expressed and nuclear restricted proteins (Stonestrom *et al.*, 2015). A number of isoforms, mainly through splicing, have been reported for all the BETs (Alsarraj *et al.*, 2013; Hnilicová *et al.*, 2013; Shao *et al.*, 2016), although functionality of the various isoforms is uncertain. Further information on the varying BET isoforms is discussed in section 3.2, with particular attention on the isoforms of BRD2. Their binding to chromatin is variable, meaning BETs can have dynamic but highly specific functions during development and homeostasis, but this can also be deleterious in a disease context (Garnier, Sharp and Burns, 2014).

## 1.7.3. BET proteins in development and homeostasis

BETs are thought to be crucial in developmental processes although the details of this are still not fully understood, especially for BRD3. It has been observed that Brd4 is associated with acetylated histones on mitotic chromosomes in early zebrafish embryos (Toyama *et al.*, 2008). Equally, there is good evidence from mouse models that these proteins are highly important in development. Homozygous knockout of *Brd2* (Shang *et al.*, 2009) or *Brd4* (Houzelstein *et al.*, 2002) in mice is embryonically lethal due to severe neural defects or lack of proper blastocyte development.

Interestingly, mice with lowered expression of Brd2 have no gross developmental defects but suffer from extreme obesity in the absence of hyperinsulinemia, suggesting altered insulin signalling and enhanced adipogenesis (Wang *et al*. 2010; Zang *et al*. 2013). BRDT is also crucial for the normal development of male germ cells, hence the interest in BRDT inhibition as a form of male contraception (Matzuk *et al.*, 2012). Overexpression studies are less common, but increased BRD2 expression specifically in lymphocytes causes B-cell lymphoma and leukaemia (Greenwald *et al.*, 2004) and it has been observed that overexpression of BRD4 in human cancer tissues correlates with a poorer prognosis (Zhang *et al*. 2015; Hu *et al*. 2015). Characterisation of BRD3 function in mouse models has not yet been published.

***Table 1.2:*** **Summary of BET manipulation in mouse models**

| | **Homozygous knockout** | **Partial knockdown (lo)** | **Overexpression** |
|---|---|---|---|
| **Brd2** | Embryonically lethal | Extreme obesity with absence of hyperinsulinemia | Specifically in lymphocytes causes B-cell lymphoma/leukaemia |
| **Brd3** | - | - | - |
| **Brd4** | Embryonically lethal | - | - |

Redundancy between BETs is apparent; for example in the context of erythrocyte development through GATA1 engagement, Brd2 depletion limits development, Brd3 depletion has no effect, whilst depletion of both Brd2 and Brd3, or Brd4 alone, completely prevents erythrocyte development (Stonestrom *et al.*, 2016). Equally, BRD2 and BRD4 have been shown to co-regulate Th17 cell differentiation through functionally independent mechanisms, BRD2 through direct interaction with STAT3, and BRD4 through indirect interactions with RNA pol II (Cheung *et al.*, 2017).

### 1.7.4. BET proteins in disease and their therapeutic inhibition

BET proteins, in particular BRD4, have been heavily studied in two main disease themes: cancer and inflammation. Firstly, in the context of cancer, aberrant translocation events have been discovered in the nuclear protein in testis gene (*NUTM1*), causing the formation of BRD3 or BRD4 NUT fusion proteins (French *et al.*, 2008) These BET oncogene fusion proteins can then drive highly aggressive, squamous cell carcinoma known as NUT carcinoma through blocking of differentiation pathways and uncontrolled cell cycling (French *et al.*, 2008). Additionally, BRD4 has been found to mediate the transcription of the oncogene *MYC*, which is a key driver of numerous cancers (Delmore *et al.*, 2011). BET proteins are also known to be important for the inflammation response with BRD2 and BRD4 recruited to inflammatory gene promoters (Belkina, Nikolajczyk and Denis, 2013) and BRD4 associated super-enhancer formation driven by NF-κB activity (Xu and Vakoc, 2014). In this way, BETs have been associated with chronic inflammatory responses that are deleterious *in vivo*, such as the cytokine storm seen in sepsis (Nicodeme *et al.*, 2010).

The association of BET proteins with a variety of diseases has driven the development of small molecule BRD inhibitors, which were first developed to target the BRDs of CBP as it was found to bind to, and modulate the activity of the tumour associated gene p53 (Sachchidanand *et al.*, 2006). However, shortly afterwards focus shifted towards inhibitors targeting BRDs of BETs. The Bradner lab has been at the forefront of this development, creating the original JQ1 compound (Filippakopoulos *et al.*, 2010). Since then, various modifications to this structure have been developed to increase stability and potency (Garnier, Sharp and Burns, 2014). Somewhat misleadingly, these inhibitors are often referred to as BRD4 inhibitors, suggesting that the effects of treatment are exclusively due to BRD4, when in fact they are the result of a pan-BET inhibition. Seven BET inhibitors are now in clinical trials for cancers, including NUT carcinoma, triple negative and oestrogen-receptor positive breast cancer, castration resistant prostate cancer, and multiple myeloma (Andrieu, Belkina and Denis, 2016)

Due the broad effects of anti-inflammation and anti-proliferation after BET inhibition, these drugs are now being tested in a range of contexts. Beyond cancer (**Table 1-3**), the effect of BET protein inhibition has been investigated in a range of other diseases including auto-immunity, infection, inflammation and fibrosis (**Table 1.4**). Of particular interest to this project is the beneficial effects seen in fibrosis models, including reduced cell proliferation, reduced expression of ECM and cytokines and a lessened myofibroblast phenotype (Tang *et al.*, 2013a; Ding *et al.*, 2015; Zhou *et al.*, 2017)

## *Table 1-3* - BET inhibitor cancer literature (as correct in April 2017)

| Tool | Host | Cell type | Effect seen | Reference |
|---|---|---|---|---|
| **JQ1** | Human<br>Mouse | Prostate cancer cell line<br>Prostate cancer model | Reduced proliferation and oncogenic transcription<br>Reduced tumour size | Asangani *et al*, 2014 |
| **JQ1**<br>**RVX2135** | Mouse | Myc-induced lymphoma cells | Induced cell cycle arrest and cell death | Bhadury *et al*, 2014 |
| **I-BET151**<br>**I-BET762** | Human<br><br>Mouse | Myeloma cell lines<br>Primary myeloma cells<br>Myeloma model | Induced cell cycle arrest and apoptosis<br><br>Decreased proliferation and increased survival | Chaidos *et al*, 2014 |
| **JQ1** | Human<br><br>Mouse | Primary leukaemia cells<br>Leukaemia cell lines<br>Leukaemia model | Induced cell cycle arrest<br>Reduced tumour growth | Costa *et al*, 2013 |
| **JQ1**<br>**OTX015** | Human | Leukaemia cell lines | Induced cell cycle arrest and apoptosis | Coudé *et al*, 2015 |
| **JQ1** | Human | Triple negative breast cancer cells (TNBC)<br>TNBC xenograft | Reduced growth, angiogenesis and hypoxia response | da Motta *et al*, 2017 |
| **I-BET151** | Human<br>Mouse | Leukaemia cell lines | Induced cell cycle arrest and apoptosis | Dawson *et al*, 2012 |
| **JQ1** | Human<br>Mouse | Multiple myeloma cell lines<br>Multiple myeloma model | Reduced MYC driven transcription<br>Induced cell cycle arrest and senescence | Delmore *et al*, 2011 |
| **ABBV-075** | Mouse | Castration resistant protease cancer model | Reduced proliferation and transcription activation | Faivre *et al*, 2017 |
| **JQ1** | Human<br>Mouse | NUT cancer cells<br>NUT cancer model | Induces differentiation and cell cycle arrest<br>Reduced tumour growth and increased survival | Filippakopoulos *et al*, 2010 |
| **I-BET151** | Human | Melanoma cell lines | Induced cell cycle arrest and apoptosis | Gallagher *et al*, 2014 |
| **MS417** | Human<br><br>Mouse | Primary colon epithelial cells<br>Colon cancer cell lines<br>Liver metastasis model | Reduced epithelial-mesenchymal transition<br>Reduced proliferation, migration and invasion<br>Reduced tumour growth and metastasis | Hu *et al*, 2015 |
| **CG13250** | Human<br>Mouse | Multiple myeloma cells<br>Multiple myeloma model | Reduced proliferation and induced apoptosis<br>Prolonged survival | Imayoshi *et al*, 2017 |
| **JQ1**<br>**OTX015** | Mouse | Glioblastoma model | Reduced proliferation and increased cell death and stress response | Ishida *et al*, 2017 |
| **JQ1** | Human | Human colon cancer cell line | Downregulated c-myc and decreased growth | Kitazawa *et al*, 2017 |
| **I-BET151** | Mouse | Medulloblastoma model | Reduced oncogenic Hedgehog signalling<br>Reduced tumour growth | Long *et al*, 2014 |
| **JQ1** | Human | Leukaemia, glioblastoma and lung cancer cell lines | Reduced super-enhancer induced oncogenic transcription | Lovén *et al*, 2013 |
| **JQ1** | Mouse | Leukaemia cell line | Reduced Stat5 driven transcription | Pinz *et al*, 2015 |
| **JQ1** | Human<br>Mouse | Primary ovarian cancer cells<br>Ovarian cancer cell lines<br>Ovarian cancer model | Reduced proliferation and induced senescence and apoptosis<br>Reduced oncogenic metabolism and tumour growth | Qiu *et al*, 2014 |
| **MS436**<br>**MS417** | Human<br>Mouse | Melanoma cell lines<br>Melanoma model | Reduced proliferation and tumour growth | Segura *et al*, 2013 |
| **CPI-0610** | Human<br>Mouse | Multiple myeloma cells<br>Multiple myeloma model | Induced cell death<br>Improved survival | Siu *et al*, 2017 |
| **OTX015** | Human<br>Mouse | Lung carcinoma cell lines<br>Lung carcinoma models | Reduced proliferation and induced cell cycle arrest<br>Decreased 'stemness' markers<br>Reduced tumour growth | Riviero *et al*, 2016 |
| **JQ1** | Human<br>Mouse | Ovarian cancer cell line<br>Ovarian cancer model | Reduced pro-oncogenic PD-L1 signalling | Zhu *et al*, 2016 |

***Table 1.4* - BET inhibitor non-cancer literature (as correct in April 2017).**

| Tool | Host | Cell type | Effect seen | Reference |
|---|---|---|---|---|
| **JQ1** | Mouse | Osteoporosis model | Reduced osteoclast differentiation and activity | Baud'huin *et al*, 2016 |
| **I-BET762** | Mouse | Primary $CD4^+$ T cells<br><br>Neuroinflammation model | Reduced pro-inflammatory and increased anti-inflammatory gene transcription<br>Reduced T-cell mediated macrophage recruitment | Bandukwala *et al*, 2012 |
| **I-BET151** | Mouse | Macrophage cell line | Reduced LPS induced IL-6 production | Barrett *et al*, 2014 |
| **JQ1** | Mouse | Primary macrophages<br>Sepsis model | Reduced LPS induced cytokine production<br>Reduced LPS induced mortality | Belkina *et al*, 2013 |
| **I-BET151** | Human | Primary monocytes | Reduced cytokine induced transcription<br>Reduced downstream cytokine signalling | Chan *et al*, 2015 |
| **JQ1** | Rat | Pancreas cell line | Increased insulin secretion | Deeney *et al*, 2016 |
| **JQ1** | Mouse | Primary hepatic stellate cells<br>Liver fibrosis model | Reduced proliferation and induced senescence<br>Reduced pro-fibrotic transcription and differentiation<br>Reduced disease progression | Ding *et al*, 2015 |
| **RVX 208** | Human | Human clinical trials | Reduced major adverse cardiovascular events | Ghosh *et al*, 2017 |
| **JQ1** | Human<br>Mouse | Endothelial cell line<br>Angiogenesis model | Reduced VEGF-induced angiogenesis and vascular permeability | Huang *et al*, 2015 |
| **JQ1**<br>**PFI-1** | Human | Primary lung epithelial cells | Reduced IL-1β induced inflammation | Khan *et al*, 2014 |
| **I-BET151** | Human | Primary rheumatoid arthritis synovial fibroblasts | Reduced cytokine and TLR ligand induced inflammation and MMP production | Klein *et al*, 2014 |
| **JQ1** | Mouse | Lung disease model | Reduced inflammation through upregulation of SIRT1 | Kokkola *et al*, 2015 |
| **JQ1** | Mouse | Acute retinal ganglion cell death model | Prevented cell death and reduced inflammatory cytokines | Li *et al*, 2017 |
| **JQ1** | Mouse | Alzheimer's model | Reduced splenomegaly and neuroinflammation | Magistri *et al*, 2016 |
| **JQ1** | Human | Primary COPD alveolar macrophages and PBMCs | Reduced LPS induced cytokine production | Malhotra *et al*, 2017 |
| **JQ1** | Human<br>Mouse<br>Mouse | Primary T cells<br>Primary T cells<br>Autoimmune models | Reduced IL-17 driven cytokine production<br><br>Reduced Th17 cell differentiation | Mele *et al*, 2013 |
| **JQ1** | Mouse<br>Mouse | Macrophage-like cell line<br>Periodontitis model | Reduced LPS induced cytokine and osteoclast production | Meng *et al*, 2014 |
| **JQ1** | Human | Primary airway smooth muscle cells<br>Monocyte cell line | Induced Nrf2-dependent transcription<br>Reduced $H_2O_2$ induced ROS production | Michaeloudes *et al*, 2014 |
| **JQ1** | Mouse | Psoriasis model | Reduced RORC/IL-17A induced inflammation | Nadeem *et al*, 2015 |
| **I-BET151** | Mouse | Primary macrophages<br>LPS/inflammation model | Reduced LPS induced inflammation | Nicodeme *et al*, 2010 |
| **I-BET151**<br>**JQ1** | Human<br>Zebrafish | Chondrogenic cell line<br>Zebrafish model | Suppressed chondrocyte differentiation<br>Decreased zebrafish growth | Niu *et al* 2016 |
| **I-BET762**<br>**JQ-1** | Human | Primary airway smooth muscle cells | Reduced TGF-β1 induced cytokine production and proliferation | Perry *et al*, 2015 |
| **JQ1** | Rat | Primary myocytes | Reduced pathological cardiac hypertrophy | Spiltoir *et al*, 2013 |
| **JQ1** | Rat | Heart injury model | Reduced injured induced TLR4/TRAF6/NF-κB signalling | Sun *et al*, 2015 |
| **JQ1** | Human | Primary lung fibroblasts | Reduced proliferation, migration, cytokine production, ECM and α-SMA expression | Tang *et al*, 2013b |
| **JQ1** | Human<br>Mouse | Primary fibrotic lung fibroblasts<br>Lung fibrosis model | Reduced proliferation, migration and cytokine production<br>Reduced inflammation and disease severity | Tang *et al*, 2013a |
| **JQ1** | Mouse | Influenza model<br>Colitis model | Reduced inflammatory nitric oxide production | Wienerroither *et al*, 2013 |
| **I-BET151** | Human<br><br>Mouse | Primary renal fibroblasts and epithelial cells<br>Renal fibrosis model | Reduced ECM and α-SMA expression<br>Reduced fibrotic signalling<br>Reduced fibroblast activation and macrophage infiltration | Xiong *et al*, 2016 |

## 1.8. Project overview and objectives

The overarching objective for this project is to explore the effect of BET proteins in a keloid scar context, with particular focus on dermal fibroblasts, given that this cell type drives the bulk of keloid pathology. We have access to a pre-clinical BET inhibitor from our collaborators at GSK, called I-BET151, and will be using this compound to interrogate the function of BET proteins in dermal fibroblasts. The three main aims are to firstly examine BET expression in keloids and then to explore the effect of BET inhibition, with particular attention to the impact on IL-6 signalling, which is likely to be highly relevant for therapeutic targeting in keloids as IL-6 is frequently reported to be a key biomarker and possible mediator of keloid pathology (**Figure 1.15**).

We hypothesise, given the reported anti-proliferative and anti-inflammatory effects of BET inhibition, that we may be able to mediate key drivers of pathology including cell cycling and IL-6 production in keloid fibroblasts. The effect of BET inhibition will also be investigated in other potentially important cellular parameters that have not yet been explored in the literature, including contraction and protease activity.

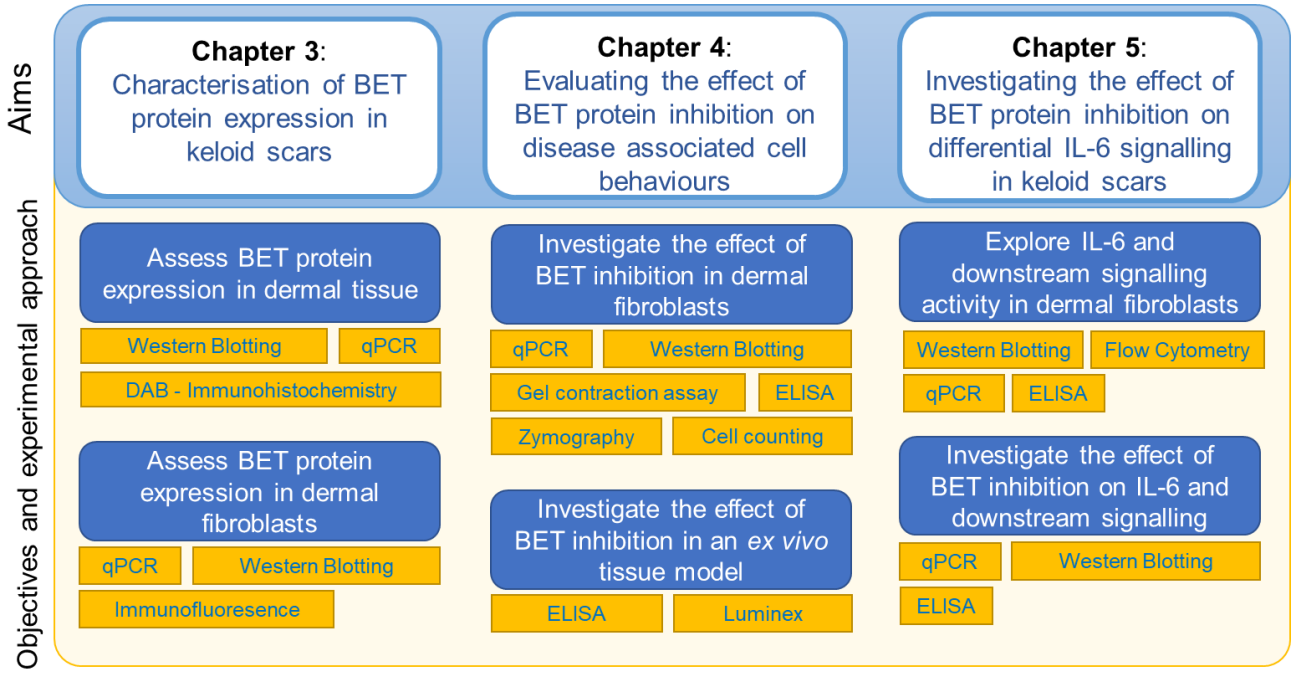



***Figure 1.15*** **- Overview of project aims and objectives with the experimental approaches to be used.**

# Chapter 2: Material and Methods

## 2.1. Cell and tissue culture methods

### 2.1.1. Tissue collection

Normal skin, normal scar and keloid scar tissue were collected from consenting patients with the assistance of Mr Soldin, St George's Hospital London and Dr Mallipeddi, Mr-Mackenzie-Ross and Mr Ng, Guys and St Thomas' Hospital under Study Title: The Molecular Mechanisms of Tissue Repair; REC Ref 14/NS/1073. The study was institutionally sponsored by St George's University London, and institutionally approved at KCL/GSTT (R&D ref RJ115/N032). Where possible, a patient information sheet was completed and collected alongside the sample to give important information about patient and scar details (example in Appendix, **Figure 7.1**).

Samples were promptly processed in the tissue culture lab at King's to preserve tissue characteristics and cell viability. Primary cells were isolated from the tissue by an enzymatic digestion or *ex vivo* culture method, as subsequently described.

### 2.1.2. Primary dermal cell isolation by enzymatic digestion

Whole tissue, including epidermis and dermis, was trimmed of fat and cut into small pieces (~1cm$^2$) before being disinfected in a series of washes of iodine and 70% ethanol. The tissue was then incubated in 0.1mg/ml Primocin (InvivoGen) in Hank's Buffered Salt Solution Modified (HBSS) for 30 minutes at RT in order to remove any fungal and bacterial contamination, including mycoplasma.

Tissue was incubated with Dispase II diluted in HBSS to a final concentration of 100µg/ml for 1 hour at 37°C. After this incubation, the epidermis was removed and the remaining dermis was finely minced (~1mm$^2$) and incubated in an enzyme digestion mix from the Miltenyi Skin Dissociation Kit overnight at 37°C.

After 12-18 hours of incubation, the digested suspension was filtered through a 100µm cell strainer in order to remove debris, and cells were counted with a haemocytometer. Cells were either used immediately, or cryopreserved. Cells were cryopreserved by centrifugation at 400xg for 5 minutes and resuspension in freezing media in cryovial aliquots of ~1 X 10$^6$ cells.

Freezing media comprised of 40% v/v foetal calf serum (FCS), 10% v/v dimethyl sulfoxide (DMSO) and 50% complete DMEM (cDMEM), which consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v foetal calf serum, 4mM L-glutamine (L-glut) and 1% v/v penicillin-streptomycin (P/S) giving a final concentration of 100 units/ml penicillin and 0.1mg/ml streptomycin. Cell suspensions were gently frozen at -80°C in a Mr Frosty™ freezing container containing isopropanol, before being moved into liquid nitrogen for long term storage.

### 2.1.3. Primary dermal fibroblast isolation by explant culture

Firstly, wells of a 6 well plate were coated with 0.1% gelatin in sterile water for ~1 hour before aspiration of the liquid and washing with PBS (10mM $PO_4^{3-}$, 137mM NaCl, 2.7mM KCl). 4mm whole tissue biopsies, including dermis and epidermis, were taken from the sample using a disposable biopsy punch and cut into 9-12 pieces. These tissue pieces were then carefully placed into the coated wells. 1ml of high serum cDMEM (20% FCS) was then added to each well. Media was changed every day thereafter with media volume increased to 2ml once cells were observed to be migrating out of the tissue. Keratinocytes were initially observed to migrate out of the tissue but were then outgrown by fibroblasts by ~2 weeks of culture. After confluency was reached, cells were pooled and split into 2 x T75 flasks for around 1 week of culture and then split into 3 x T175 flasks for a further 1 week of culture in normal serum levels (10%). Once confluency was reached, these 3 x T175 flasks were cryopreserved, as previously described, into 15 cryovial aliquots.

### 2.1.4. Ongoing primary dermal fibroblast culture

Fibroblasts were seeded and maintained in cDMEM (see above). Frozen aliquots of cells were quickly defrosted in a water bath before seeding into a T75 flask with 10ml of cDMEM pre-warmed at 37°C. Media was changed at ~ 24 hours or after cell adherence and then every 3-4 days until the flask reached ~80% confluency. Cells were split at this point in order to avoid effects of contact inhibition (Abercrombie, 1970). Cells were split by removing the media, washing with PBS and incubating with Trypsin-EDTA solution, 1ml or 3ml for a T75/T175 flask respectively, for 5-8 minutes at 37°C. After cells were observed to be in a single cell suspension, cDMEM was added in order to neutralise enzymatic activity and the resulting cell suspension was used to continue passaging of the cell line. Typically cells were split 1:4-1:10 in order to reach confluency in 3-7 days. Culturing of cells was continued until passage 10 when

the effects of *in vitro* culturing were thought to be significant (Allahbakhshian-Farsani *et al.*, 2014).

### 2.1.5. *Ex vivo* tissue culture

Due to the volume of collagen needed, a stock of collagen I was created. Rat tails obtained from surplus adult rats at King's College London Biological Services Unit were soaked in 70% ethanol before dissection of tendons of pure collagen I. 1.5g of tendons were washed extensively in DI water and added to 100ml of 0.05% glacial acetic acid in deionised (DI) water supplemented with 2.5µg/ml of Amphotericin B to prevent fungal growth. This mixture was stirred gently at 4°C for at least two weeks.

The resulting viscous liquid was then centrifuged at 3000xg for 15 minutes, before this supernatant was further centrifuged at 35,000xg for 1 hour at 4°C. The collagen solution was kept in a sterile duran bottle for up to 6 months at 4°C. The collagen I preparation was run on an SDS-PAGE gel and stained with Coomassie Blue in order to compare to a commercially available collagen I solution (**Figure 2.1**), which indicated a very similar staining pattern and therefore a comparable protein composition and quantity.

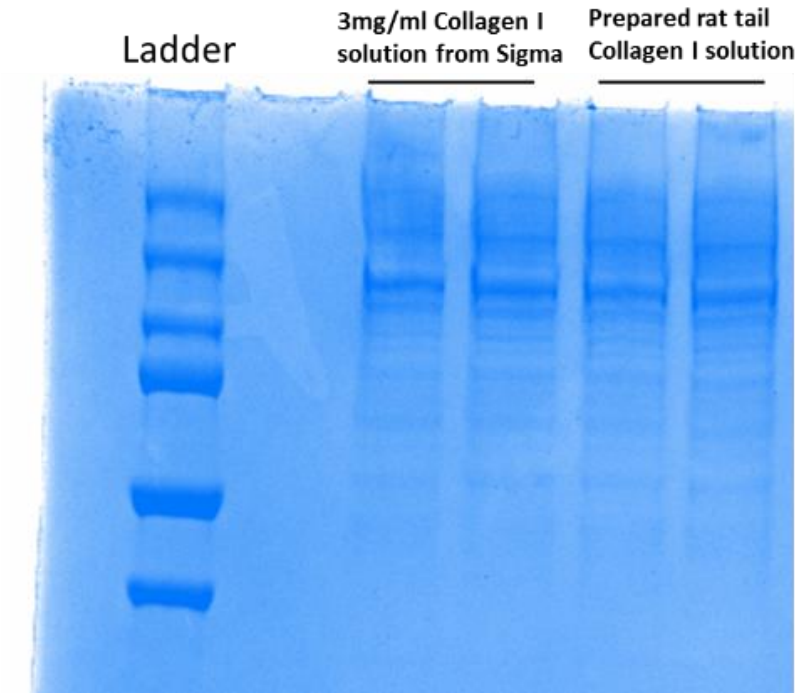



***Figure 2.1*** **- Coomassie Brilliant Blue stained SDS-PAGE gel examining lab-prepared collagen I solution.**

To set up the *ex vivo* tissue model as published in (Bagabir *et al*. 2012b), 4mm whole tissue punch biopsies, including epidermis and dermis, were taken from normal and keloid tissue and soaked for at least 30 minutes in HBSS supplemented with 0.1mg/ml Primocin to remove contaminating microorganisms. Media was comprised using William's E media supplemented with 0.1mg/ml insulin, 10ng/ml hydrocortisone, 4mM L-glut and 1% v/v P/S, giving a final concentration of 100 units/ml penicillin and 0.1mg/ml streptomycin.

A base layer of media-gel was set into a 24 well plate using 250µl media with 1µM I-BET151 or DMSO vehicle, 250µl collagen I solution and 4µl of 1M NaOH and incubated for ~15 minutes at room temperature. Once this layer was set, a tissue biopsy was added to the set media-gel layer and further media-gel solution was added to embed the tissue leaving the epidermis at the air liquid interface (ALI) (**Figure 2.2**)

Once set, 200µl of William's E media containing 1µM I-BET151 or DMSO vehicle was added to the surface of the gel. This media was collected at 24 hours for supernatant analysis and refreshed every 2-3 days thereafter. After 1 week of culture biopsies were fixed in 4% paraformaldehyde (PFA) or stored in RNAlater at -20°C for future RNA and protein analysis.

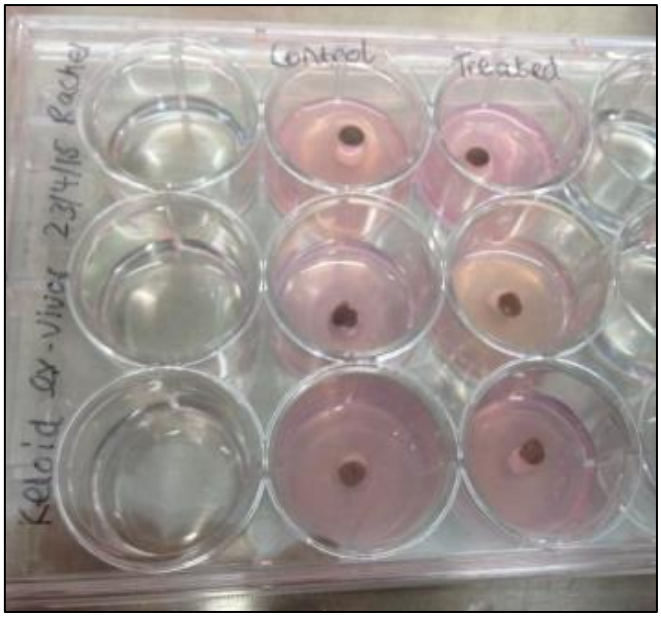



***Figure 2.2* - Photograph of *ex vivo* tissue culture set up as published in Bagabir *et al*, 2012b.** 4mm keloid biopsies are set-up in technical triplicate with control (0.01% DMSO) or treated (1µM I-BET151) and incubated for 1 week.

## 2.2. Fibroblast functional phenotype experiments

### 2.2.1. Pharmacological BET inhibition

The BET proteins were pharmacologically inhibited through the use of I-BET151, a small molecule inhibitor compound from GlaxoSmithKline. This compound was made up in DMSO to a stock concentration of 10mM. Dose dependent experiments were carried out but in general practise a dose of 1µM was used with a vehicle control of 0.01% DMSO.

### 2.2.2. Cell counts for proliferation

Typically, cells were seeded into 6 well plates at a concentration of 1-5 X $10^4$ cells/well in 2ml media. After adherence (~5 hours later) media was removed and replaced with either inhibitor or vehicle supplemented media. Media was refreshed every 24 hours in order to maintain optimum inhibitor efficacy. After 72 hours of culture, cells were trypsinised and counted in technical duplicate using a haemocytometer or multi-chamber FastRead counting slides.

### 2.2.3. Contraction gel assay

Fibroblasts were seeded into collagen gels by mixing 500µl of cell suspension at 1.5 x $10^5$/ml, 200µl of collagen I solution and 6µl of 1M NaOH in order to neutralise and set the gel. This suspension was dispensed into a well of a 24 well plate and left for ~15 minutes at room temperature to set. The collagen gel was then released from the edge of the well with a p20 pipette tip in order to examine contraction potential in free floating gels. 24 well plates holding collagen gels were imaged at 72 hours using the BioRad Gel Doc+ imaging system. Collagen gel areas for each well were then quantified using ImageJ.

## 2.3. Protein assays

### 2.3.1. Protein lysis and western blotting

Lysates were prepared from cell pellets or homogenised de-epithelised tissue diluted into radioimmunoprecipitation (RIPA) buffer (50mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150mM NaCl and 1mM EDTA) with freshly added protease and phosphatase inhibitors (**Table 2.1**)**.** Lysates were incubated on ice in 1.5ml microcentrifuge tubes for 20 minutes with frequent vortexing before being centrifuged at 10,000 x g for 5 minutes to pellet cell debris. The resulting lysate supernatants were moved to new 1.5ml microcentrifuge tubes.

***Table 2.1*** **- Components added to RIPA buffer to create protein lysis buffer** (all sourced from Sigma-Aldrich).

| | Final concentration | Purpose |
|---|---|---|
| **Leupeptin** | 1µg/ml | Protease inhibitor |
| **Apropeptin** | 1µg/ml | Protease inhibitor |
| **Pepstatin** | 1µg/ml | Protease inhibitor |
| **NaF** | 1mM | Phosphatase inhibitor |
| **$Na_3VO_4$** | 1mM | Phosphatase inhibitor |
| **PMSF** | 1mM | Protease inhibitor |

Lysate protein levels were quantified using a BCA kit (Santa Cruz). Briefly, 25µl of each sample, diluted 1:5 in PBS, and 25µl of a BSA standard dilution was added in duplicate to each well of a 96 well plate. The plate was incubated for 30 minutes at 37°C. A colour change from green to purple indicated presence of protein. Colour intensity was then measured on a spectrophotometer plate reader at 562nm and Microsoft Excel was used to make a standard curve and subsequently calculate protein concentrations in the tested samples. Samples to be analysed by western blot were made up to a defined quantity of protein (5-15µg) in a total volume of 20-25µl in water including 4-5µl 5X loading buffer (0.25% bromophenol blue, 0.5M DTT, 50% glycerol, 10% SDS in 0.25M Tris-HCl, pH 6.8). These samples were frozen at -20°C until use, if not run immediately.

Prior to running the western blot, samples were briefly boiled to denature the sample and then cooled before being loaded onto precast gradient gels from Nugen or BioRad, with 7µl of BioRad protein standard ladder in order to identify protein size. Gels were run at 120V-200V for 40-120 minutes in order to separate protein bands of interest. Gels were then transferred in transfer buffer (25mM Tris-HCl, 200mM glycine, 20% ethanol) at 112V for 90 minutes onto nitrocellulose membrane. After transfer, membranes were briefly stained with Ponceau Red solution (0.1% w/v Ponceau S in 5% acetic acid) in order to identify lanes and check successful transfer. Membranes were trimmed and cut where appropriate and blocked in 5% skimmed milk powder in TBS (20mM Tris, 150mM NaCl, pH 7.6) with 0.1% Tween 20 (TBS-t) for 1 hour at room temperature to prevent non-specific antibody binding. After blocking, primary antibody. generally diluted 1:1000 in antibody diluent (0.02% sodium azide, 1% BSA in TBS-t), was added to blots in sealed plastic packets and incubated on 4°C rocker overnight. As well as the protein of interest, a loading control (GAPDH/HDAC1/α-tubulin) was included to confirm equal protein loading.

After primary antibody incubation, blots were washed in TBS-t and incubated with HRP-conjugated secondary antibody diluted in 1:5000 in TBS. The secondary antibody species was targeted to the host species of the primary antibody, either rabbit or mouse (Jackson ImmunoResearch Laboratories, Inc.). Blots were incubated with secondary antibody on the room temperature rocker for 1 hour. After incubation, blots were washed extensively with TBS-t, followed by a final TBS wash. The Clarity™ Western ECL substrate kit (Bio-Rad) was used to visualise antibody binding. The peroxidase and luminol reagents were mixed 1:1 and a small amount (~1ml for full size membrane) was added to the blot and incubated for 5 minutes at room temperature. Chemiluminscent images were captured on the BioRad Gel Doc+ system for between 2 and 20 minutes, as well as a light photograph for overlay of the protein size ladder to determine band weights.

Band sizes of the protein of interest and the loading control were quantified in each sample using the BioRad ImageLab 3.1 software, allowing normalisation and calculation of relative protein expression.

***Table 2.2* - Antibodies used in Western Blotting.**

| Target | Species | Mono/poly-clonal | Supplier |
|---|---|---|---|
| GAPDH | Rabbit | Poly | Abcam |
| α-tubulin | Mouse | Mono [DM1A] | Abcam |
| α-SMA | Rabbit | Poly | Abcam |
| pStat3 *(Thy705)* | Rabbit | Mono [D3A7] | CST |
| Stat3 | Rabbit | Mono [D3Z2G] | CST |
| pAkt *(Ser473)* | Rabbit | Mono [D9E] | CST |
| Akt | Rabbit | Poly | CST |
| pErk1/2 *(Thr202/Tyr204)* | Rabbit | Mono [D13.14.4E] | CST |
| Erk1/2 | Rabbit | Mono [137F5] | CST |
| BRD2 | Rabbit | Poly | GSK |
| BRD3 | Rabbit | Poly | GSK |
| BRD4 | Rabbit | Poly | GSK |
| Ki67 | Rabbit | Mono [SP6] | Abcam |

([clone]. CST = Cell Signaling Technology, GSK = GlaxoSmithKline)

### 2.3.2. Enzyme linked immunosorbent assay (ELISA)

The production of IL-6 and sIL-6r was measured by Human DuoSet ELISA, from R&D Systems as per manufacturer's protocol, in high binding 96 well plates.

Briefly, plates were coated with capture antibody diluted in PBS as per lot instructions, plates were sealed and incubated at room temperature overnight. The following day, wells were blocked with 300µl reagent diluent (1% BSA w/v in PBS) for 1 hour before three washes with wash buffer (0.05% Tween in PBS). A standard curve was made by re-suspending the supplied lyophilised standard as per lot instructions, then serially diluting 2-fold in reagent diluent to create a seven-point standard curve. 100µl of duplicate standards and 100µl of samples (plated neat and 1:10 diluted in reagent diluent) were added to the plate, sealed and incubated for 2 hours at room temperature. 100µl of detection antibody diluted in reagent diluent, as per lot instructions, was added to wells after three washes with wash buffer and incubated for 2 hours at room temperature. Streptavidin-HRP was diluted 1:40 in reagent diluent and 100µl was added to wells after three washes with wash buffer and incubated for 30 minutes. TMB substrate was created by mixing a 1:1 solution of Reagent A and B from the R&D kit and 100µl was added to each well after three washes and incubated at room temperature. The addition of TMB substrate caused a gradual colour change to blue, and this reaction was terminated after 5 – 20 minutes when the lowest standard appeared bluer than the negative control well. The reaction was stopped with the addition of 50µl of 0.16M sulfuric acid per well and colour intensity was measured at 420nm on a spectrophotometer plate reader.

A standard curve was constructed by plotting the $\log_{10}$concentration against absorbance and a 4 parameter logarithmic curve was fitted to the data. Absorbances for samples were interpolated as unknown X values, inverse-logged and multiplied by the dilution factor, if appropriate, to calculate the level of analyte in the sample in pg/ml.

### 2.3.3. Luminex Analysis

Luminex is another antibody based technology to detect analytes in solution, however Luminex has the advantage over ELISA that antibodies can be multiplexed in order to simultaneously detect multiple analytes in the same experiment. A magnetic bead Luminex kit from R&D Biosystems was used for this analysis to measure MMP1, MMP3, MMP7, MMP8, MMP9, MMP10, MMP11, MMP12, MMP13 and another kit to test for analytes TIMP1, TIMP2, TIMP3 and TIMP4 in 24 hour supernatants from *ex vivo* models and fibroblast cultures +/- I-BET151. The kit was able to detect both pro and activated forms as well as MMP-TIMP complexed forms of protein.

The kit supplied a mix of magnetic beads each coated with antibodies specific to an analyte of interest and labelled with a unique dye on the red to far-red spectrum. The kit also supplied a standard mix of all analytes which was diluted 3 fold to produce a 6 point standard curve to estimate concentration of unknown samples as in an ELISA. Samples were diluted 1:10 and 1:50 in order to capture the variation in possible analyte expression between the different samples.

The protocol was then followed as instructed by the product data sheet. Briefly, standards and diluted samples were incubated with the magnetic bead mix in a 96 well plate format for 2 hours at room temperature on an orbital shaker protected from light. A magnetic device designed for 96 well plates was then used to hold the beads to the bottom of the plate to allow washing of beads in wash buffer (0.05% Tween in PBS). 3 x 1 minute washes were conducted, removing the magnet before the addition of new wash buffer to ensure thorough washing of the beads. Biotinylated secondary antibody mix was then added to the beads and incubated for an hour in the same conditions before another 3 washes. Streptavidin-PE was then added to the beads for a further 30 minutes in the same conditions before another 3 washes and a final resuspension in wash buffer.

Plates were then read using a Luminex analyser (Flexmap 3D) which acquired 50 events per analyte in each well with a fast flow rate (60µl/minute). This analyser uses flow cytometry to singularise beads and lasers are used to identify the bead colour (to identify the analyte) and the streptavidin reporter intensity to quantify the secondary signal (to identify the quantity of analyte that has bound). Results are analysed in a very similar way to how ELISA data is processed, however a 5-parameter curve is used as this type of curve fitting makes fewer mathematical assumptions, which is better in such a diverse data set with multiple analyte measurements.

### 2.3.4. Zymography assay

7µl fibroblast supernatant was incubated with 10µl of 10x assay buffer (500mM HEPES, 100mM CaCl2, 0.5% Brij-35, 10mM DTNB [also known as Ellman's reagent], pH to 7.0), 5mM thiopeptide (pan-MMP chromogenic substrate, Enzo Biosciences) and 90µl of water in triplicate in a 96 well plate. Media only and water only wells were also included as negative controls.

Plates were incubated in the dark at 37°C and active proteases within the supernatant were able to cleave the MMP substrate, releasing a thiol group which was free to reduce DTNB causing the conversion from a colourless to a yellow-coloured substance which could be detected by spectrophotometry. Plates were read at 412nm at 0, 6, 24 and 48 hours of incubation and absorbance values were normalised to controls to give a relative value of protease activity.

### 2.3.5. Immunofluorescent staining

For immunofluorescent staining, cells were grown on poly L-lysine coated coverslips. Circular coverslips were first acid washed by gentle stirring in 1M HCl at 60°C overnight. Following acid washing, coverslips were extensively washed in DI water and then 95% ethanol where they could be stored for several months before coating. Coverslips were incubated in 0.1mg/ml poly L-lysine solution in petri dishes for 1 hour at room temperature in the tissue culture hood. Coverslips were then washed three times in PBS and dried on filter paper in the tissue culture hood before being stored in a sterile 50ml falcon tube.

Cells were seeded onto coated coverslips placed in the wells of 24 well plates. Cells were grown in the presence of 0.01% DMSO vehicle or 1µM I-BET151 supplemented cDMEM. After 24 hours of treatment, coverslips were washed with PBS before fixation in 4% PFA at 37°C for 10 minutes. Fixed coverslips were washed several times in PBS and were stored at 4°C until staining.

For immunofluorescent staining, cells were washed in PBS before incubation in 0.1M glycine in PBS to reduce background fluorescence by quenching formaldehyde cross-linking. Cells were then washed and permeabilised in 1% Triton X100 in PBS, and then washed with PBS before blocking for 30 minutes at room temperature in 10% chicken serum and 0.5% BSA in PBS. Primary Ki67 antibody (**Table 2.2**) diluted 1:50 was then added in a mild blocking solution of 1% chicken/goat serum and 0.5% BSA in PBS overnight at 4°C. Fluorescently conjugated secondary antibody was added, diluted 1:750 in the mild blocking solution at room temperature

for 1 hour protected from light. DAPI diluted 1:1000 in PBS was then added for 10 minutes at room temperature before washing and mounting onto slides with Dako Fluorescent mounting media. Slides were imaged on a Zeiss Axioplan2 fluorescent microscope and pictures were taken using the x10 objective lens. Using Image J, a count of DAPI and Ki67 positivity was taken in 3 frames of view per coverslip in order to calculate the proportion of Ki67 positivity and hence the degree of active proliferation in the population.

### 2.3.6. IL-6r flow cytometry

Flow cytometry was used to examine IL-6r expression in fibroblasts. Fibroblasts were cultured in the absence or presence of serum for 24 hours prior to trypsinisation and PBS washing. Cells were stained for IL-6r and viability by incubation in 100µl of master mix, made of FACS buffer (5mM EDTA, 5% FCS in PBS) containing 1% v/v REAfinity™ phycoerthyhin (PE) conjugated IL-6r antibody from Miltenyi Bio (clone REA291) and 0.1% v/v eBioscience™ Fixable Viability Dye eFluor™ 506. Following staining, cells were then fixed in 2% PFA for 15 minutes at 4°C prior to 3 x washes in FACS buffer as previously defined. Each sample was then split in half, where half was kept in FACS buffer in dark 4°C conditions prior to acquisition and one half was permeabilised for intracellular staining. Cells to be permeabilised were washed twice in 0.5% saponin in PBS to temporarily allow antibody internalisation by disrupting cholesterol components within the plasma membrane followed by another 30 minute incubation of 1% v/v IL-6r antibody in 0.05% saponin. 3 x FACS buffer washes were completed before all samples were re-suspended in 500µl FACS buffer and transferred to polystyrene round bottom FACS tubes.

A number of controls were needed in this process, firstly for use as an IL6r positive control 5µl of OneComp eBeads™ Compensation Beads were stained for 30 minutes alongside cells before 2 x FACS buffer washes. Negative controls were also included, to account for the autofluorescence of fibroblasts and possible size and fluorescence changes caused by permeabilisation. Therefore an unstained and Live/Dead stained only control was included for each condition. Data was acquired on the FACSCanto II flow cytometer and resulting .fcs files were analysed using Flow Jo V.10. To analyse IL-6r expression, cells were gated stringently as shown below in the representative plot generally using the Live/Dead only controls **Figure 2.3**). Briefly, events were gated on cells, singlet cells, and live cells, before IL-6r expression by PE fluorescent signal was expressed as a histogram. Overlayed histograms were used to visualise shifts in staining compared to controls or compared to different cells/conditions. A geometric mean of mean fluorescent intensity (MFI) was also calculated in FlowJo for each sample and control to quantify relative levels of expression versus background fluorescence.

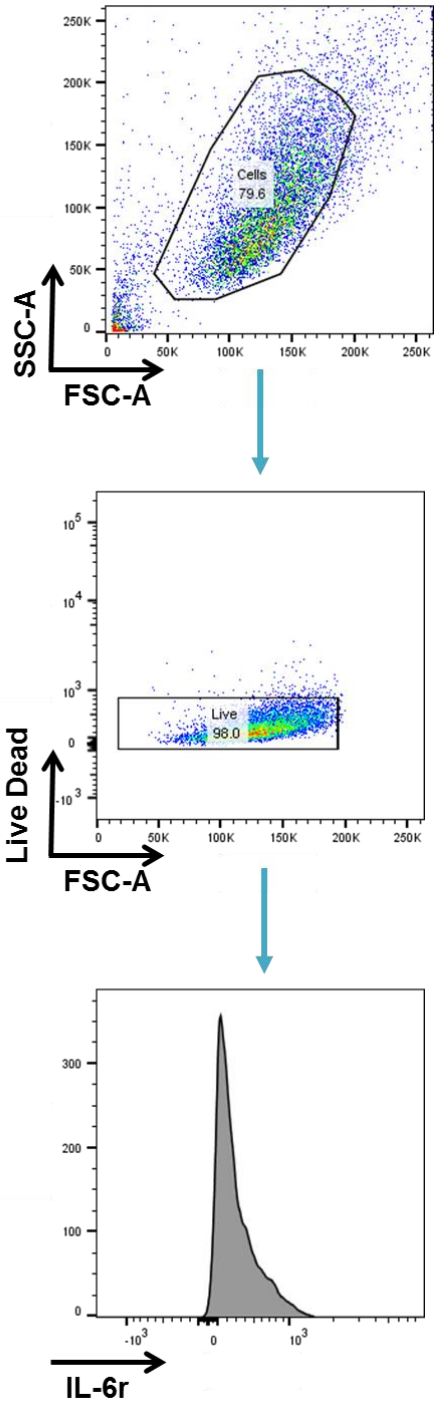



***Figure 2.3*** **- Representative gating strategy for analysis of IL-6r expression.** Events were gated on cells by SSC-A (side scatter area) and FSC-A (forward scatter area), live cells (live/dead dye APC negative) before plotting IL-6r expression (PE mean fluorescence intensity, MFI) as a histogram to analyse IL-6r levels in each sample.

## 2.4. Tissue staining

### 2.4.1. Tissue fixation, embedding and sectioning

Tissues were fixed in 4% PFA for 18-24 hours before being rinsed and stored in 70% ethanol within histology cassettes. Samples were loaded into the Leica TP1020 processing machine in the lab of Carl Hobbs, Wolfson Centre for Age Related Diseases, KCL. The machine contained 9 sequential stations:

1. 90% IMS (Industrial Methylated Spirit)
2. 100% IMS
3. 100% IMS
4. 100% IMS
5. 1:1 IMS- Xylene
6. Xylene
7. Xylene
8. Xylene
9. Paraffin wax

Samples were incubated in each station for 60 minutes and kept in molten paraffin wax until embedding. Tissue was then embedded in paraffin wax between a metal mould and histology cassette and cooled slowly to prevent wax cracking. Once set, the paraffin blocks were removed from the metal moulds and stored at room temperature.

Blocks were cooled on ice for around 5 minutes prior to sectioning and a microtome was then used to section into the block. The depth of cutting was decreased to 3-5µm once the tissue reached the face of the blade and the wax was easily ribboning. Sections were then floated in a 60°C water bath and slides were lifted to the surface of the water to attach sections. Slides were labelled, dried in a slide rack at room temperature and then baked at 60°C for 1 hour to adhere the section to the slide.

### 2.4.2. Haemotoxylin and Eosin staining

Haemotoxylin and eosin (H&E) staining was carried out on de-waxed and rehydrated sections in order to analyse general tissue morphology. Slides were de-waxed and rehydrated by 2 x 5 minutes in xylene before sequential washes in decreasing concentrations of IMS for 1 minute each before incubation in distilled water until staining. Slides were then incubated in Mayer's haemotoxylin stain for 5 minutes, rinsed in tap water until 'blued', differentiated for 30 seconds in 0.1% acid alcohol, rinsed once again in water before bluing in ammonia water and further tap washing. Slides were then incubated in eosin for 30 seconds to 1 minute before being submerged into 95% ethanol to differentiate staining and then coverslips were mounted with DPX.

### 2.4.3. Haemotoxylin Van Gieson staining

Haemotoxylin Van Gieson (HVG) staining was conducted by Carl Hobbs, Wolfson Centre for Age-Related Diseases on de-waxed and rehydrated sections as previously described. Slides were held in distilled (DI) water before treatment with iron alum for 5 minutes and washing again in DI water. Slides were then stained in Mayer's haematoxylin for 5 minutes before blueing in tap water and rinsing in DI water. Slides were then stained in Van Gieson stain for 5 minutes (1% acid fuchsin, saturated picric acid in distilled water) before rinsing in 100% IMS and coverslip mounting with DPX.

### 2.4.3. Immunohistochemistry at GSK

3,3'-diaminobenzidine (DAB) immunohistochemistry was carried out on n=10 normal skin, normal scar and keloid scar although not all samples were of a good quality to be scanned due to tissue damage during processing. The Ventana system was used to automatically stain tissues to achieve a more consistent and therefore highly comparable set of stained slides. The Ventana system could accommodate 30 slides per run.

Briefly slides were de-waxed 'offline' in Xylene as the Ventana de-waxing protocol was based on high heating and was found to damage our tissue sections substantially. Slides were therefore 'wet loaded' with PBS over the sections to keep them hydrated. Antigen retrieval was conducted with pH 8.5 Cell Conditioning Solution 1 (Ventana Medical Systems Inc.), at 95°C

for 4 cycles of 4 minutes. Slides were washed and primary BRD2 or BRD4 antibody (both polyclonal, Bethyl Laboratories Inc.) was hand applied with 50µl per slide diluted 1:200 in DISCOVERY Antibody Diluent (Ventana Medical Systems Inc.) and incubated for 32 minutes at 37°C. After further washing, Discovery™ Universal Secondary Antibody (Ventana Medical Systems Inc.) was automatically added by the machine and incubated for another 32 minutes at 37°C. After further washes, the on-board DISCOVERY DAB Map Detection Kit (Ventana Medical Systems Inc.) was used to stain antibody complexes brown to visualise BRD2 and BRD4 staining. Slides were then counterstained with Mayer's haemotoxylin before rinsing in water. After staining, slides were mounted by hand with coverslips using DPX.

### 2.4.4. Slide scanning

To capture high magnification, whole-slide images of histological staining, slide scanning was conducted using a Hamamatsu NanoZoomer 2.0-HT Whole Slide Imager located in the Centre for Stem Cell and Regenerative Medicine Research, Guy's Hospital, King's College London.

Slides were manually loaded in batches of 10 and were initially quick scanned in order for the user to draw a region of interest and to add focal points for the auto adjustment of focus by the machine. A detailed scan was then taken taking between 5 and 25 minutes per slide. Slides were saved as .ndp files and visualised in NDP.view2 software where images could be created and exported as image files.

## 2.5. Nucleic acid assays

### 2.5.1. RNA extraction from cells

For RNA extraction from fibroblasts, cells were collected in RLT buffer (350µl used for up to 1 X $10^6$ cells) and stored at -80°C until extraction. Samples were thoroughly lysed through a 20 gauge needle before extraction to maximise RNA yield. RNA was extracted using the Qiagen RNeasy mini kit with an optional step of on-column DNAse removal included in the protocol. The resulting RNA samples were eluted in 30µl RNAase-free water.

### 2.5.2. RNA extraction from tissue

RNA extraction from tissue was a similar process to above, as the Qiagen RNeasy Fibrous Tissue Mini Kit was used. 4mm whole tissue biopsies, including epidermis, were used, as a small pilot experiment showed a substantial loss in RNA integrity after a 1 hour dispase step to remove epidermis. It was also noted that the time between removal from patient and preservation in RNAlater solution needed to be minimal. Therefore, a targeted sample collection was conducted with local sources (i.e. Guy's campus) to preserve tissue as quickly as possible.

To extract RNA, a 4mm biopsy of tissue stored in RNAlater was placed into a 2ml microcentrifuge tube containing a nuclease-free stainless bead and 300µl RLT lysis buffer. Eppendorfs were then loaded and clamped into the TissueLyser II machine which oscillated samples at 30Hz for 2 x 5 minutes to homogenise tissue. The resulting tissue homogenates were incubated in Proteinase K solution (1mg/ml in PBS) at 60°C for 10 minutes to further break down the tissue, especially the fibrous keloid matrix. Lastly, these lysates were spun at full speed through QIAshredders for 5 minutes for a final homogenisation step. RNA extraction was then carried out in the same way as above following the kit instructions, and samples were eluted in 40µl of nuclease free water.

### 2.5.3. RNA Quality Analysis

To firstly quantify and assess purity of the RNA, 1µl of RNA was loaded onto the NanoDrop 2000 after nuclease water was used as a blanking solution. Purity was analysed by the A260/280 ratio, with an ideal value of 2, samples tended to range from 1.9 to 2.1 and were deemed acceptable. NanoDrop analysis also gave an estimated concentration, although the accuracy of this was thought to be decreased when the concentration fell below 20ng/ml. To analyse the integrity of the RNA that was extracted, especially that from tissue which is prone to nuclease degradation, 1.5µl of RNA was denatured at 60°C for 2 minutes before being tested using an automated capillary gel electrophoresis machine, an Agilent 2100 Bioanalyzer.

The Agilent RNA 6000 Nano kit was used, with an mRNA detection range of 25–250 ng/µl. The analysis chip was loaded with gel-dye mix, before the loading of 1µl RNA 600 Nano marker in each analysis well and then the loading of 1µl of sample RNA or 1µl of supplied RNA ladder. The chip was then mixed in the chip vortexer and loaded onto the Bioanalyzer for analysis within 5 minutes.

The experimental took around 30 minutes, producing an estimated concentration that is more sensitive than NanoDrop analysis, as well as an RNA integrity number (RIN). The RIN was automatically calculated based on the RNA electropherogram trace generated for each sample, with the ratio of 18S to 28S ribosomal RNA (rRNA) peaks being particularly important in this analysis as these should be the most abundant in the extracted RNA. RIN values change from 1 to 10, with 10 being the highest integrity and 1 being the most degraded. The electrophoresis track for each sample was also generated as an image, where degraded samples were clearly identifiable by a smeared band rather than two distinct bands (18S and 28S rRNA) (**Figure 2.4**).

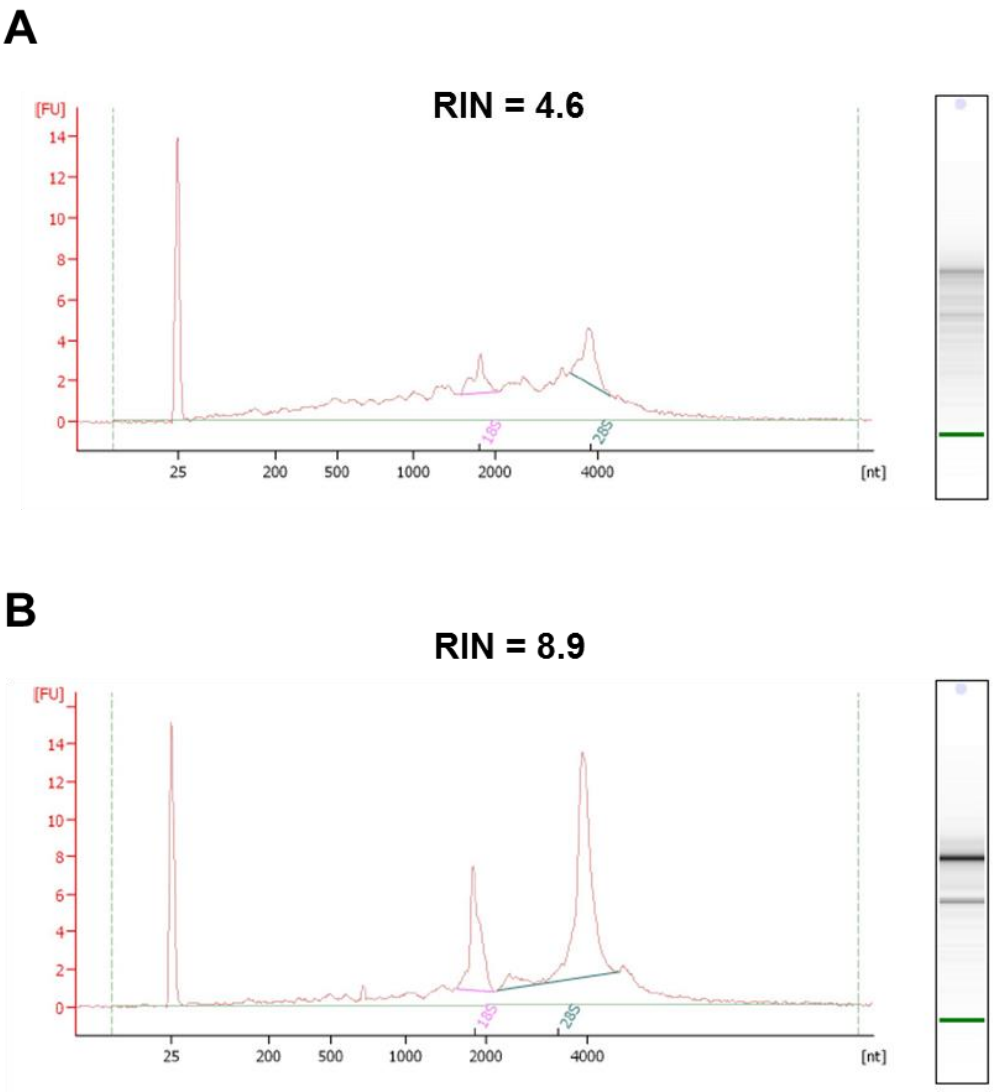



***Figure 2.4*** **- Example of Bioanalyzer results with electrophoresis image, electrogram trace and calculated RIN number.** Low (**A**) and high (**B**) integrity RNA

### 2.5.4. Reverse transcription of RNA to cDNA

Samples for reverse transcription were made up to a defined quantity of RNA (100-250ng) in nuclease-free water to a volume of 11.5µl, 1µl of random primers were then added to each sample to a final concentration of 1µM. Tubes were incubated at 65°C for 5 minutes before the addition of 7.5µl of master mix (**Table 2.3**). A thermocycler was then used to incubate the samples for 10 minutes at 25°C, 60 minutes at 42°C and then 10 minutes at 70°C before cooling on ice. The resulting cDNA was frozen at -20°C until qPCR analysis.

***Table 2.3*** **- Components of reverse transcription master mix.**

| Reverse transcription master mix (n= number of samples plus 1) | |
|---|---|
| 5X RT buffer (Thermo Scientific) | n x 4µl |
| Nuclease-free water | n x 1.5µl |
| Ribolock RNAase inhibitor (Promega) | n x 0.5µl |
| RevertAid Reverse Transcriptase (Thermo Scientific) | n x 0.5µl |
| 10µM dNTPs (Promega) | n x 2µl |

### 2.5.5. PCR primer design

Firstly, a standard curve was constructed with pooled cDNA from a range of fibroblast cultures grown under different conditions, serially diluted 5-fold in nuclease free water to create a 7 point standard curve. In practise, the 1:5 dilution was used as the 'top standard'. cDNA samples to be quantified were diluted 1:10.

A publicly available database (https://pga.mgh.harvard.edu/primerbank/) of somewhat validated PCR primers was searched for target genes. These primers were entered into NCBI Primer Blast to check specificity and amplicon size. If these primers were deemed unsuitable (e.g. amplicon size too large for qPCR), or the database returned no results, primers were designed from scratch. qPCR primers were designed using the NCBI Primer Blast website tool, an amplicon size limit of 150bp was selected with an optimum melting temperature of around 60°C. Primers were also specified to span an exon-exon junction to prevent amplification of contaminating genomic DNA. Primer sets were picked from the generated list on the basis of the lowest self-complementarity. Primers were diluted to create a 10µM forward/reverse mix and then tested against the standard curve.

***Table 2.4*** **- List of primer sequences used in this project.** (Ordered from Sigma-Aldrich at 10 nmoles).

| Gene | Forward sequence | Reverse sequence |
|---|---|---|
| ***B2M*** | TTCTGGCCTGGAGGCTATC | TCAGGAAATTTGACTTTCCATTC |
| ***ESP8*** | CGACCAAGGGGACTTTGAG | TGCCATCATCTCAGGTGTTT |
| ***P4HB*** | GGAATGGAGACACGGCTTC | TTCAGCCAGTTCACGATCTC |
| ***BRD2*** | GTCAAACTGGGTCTACCGGATT | CTTTTCCAGCGTTTGTGCCA |
| ***BRD3*** | TCA AATTGAACCTGCCGGATT | TGCATACATTCGCTTGCACTC |
| ***BRD4*** | ACAACAAGCCTGGAGATGACA | GTTTGGTACCGTGGAAACGC |
| ***BRD4L*** | CCGGAAATGAAGCCTGTGGAT | TTTTCAGGTCCTTTTTGGGCG |
| ***MMP1*** | GAGCAAACACATCTGAGGTACAGGA | TTGTCCCGATGATCTCCCCTGACA |
| ***MMP3*** | CCCTCTATGGACCTCCCCCT | ATTTGCGCCAAAAGTGCCTG |
| ***ASMA*** | CCGACCGAATGCAGAAGGA | ACAGAGTATTTGCGCTCCGAA |
| ***COL1A1*** | CTTTGCATTCATCTCTCAAACTTAGTTTT | CCCCGCATGGGTCTTCA |
| ***COL3A1*** | CCATGAATGGTGGTTTTCAG | GTGTTTAGTGCAACCATC |
| ***FN*** | TGGACCAGAGATCTTGGATGTTC | CGCCTAAAACCATGTTCCTCAA |
| ***SOCS3*** | CCTGCGCCTCAAGACCTTC | GTCACTGCGCTCCAGTAGAA |
| ***IL6*** | CCATCTTTGGAAGGTTCAGGTTG | ACTCACCTCTTCAGAACGAATTG |
| ***IL6R*** | CAGCTGAGAACGAGGTGTCC | GCAGCTTCCACGTCTTCTTGA |
| ***GP130*** | CGGACAGCTTGAACAGAATGT | ACCATCCCACTCACACCTCA |
| ***ADAM17*** | GACTCTAGGGTTCTAGCCCAC | GGAGACTGCAAACGTGAAACA |
| ***STAT3*** | ACCAGCAGTATAGCCGCTTC | GCCACAATCCGGGCAATC |

### 2.5.6. SYBR-Green RT-qPCR

Successfully designed primers were identified on the basis of specificity, by amplification of a single product by melt curve, and efficiency through a standard curve fit coefficient ($R^2$) of over 0.98. In cases where the expression of the target gene was low in fibroblast cultures, standard curves were often only linear over the first 4-5 dilutions so were still deemed acceptable for use in assays.

For each qPCR run, a master mix for each gene to be analysed was made with n x 5µl of SensiMix™ SYBR® No-Rox (BioLine) and n x 0.2µl of 10µM forward/reverse primer mix, where n = number of samples and standard tubes plus 1. 5.2µl of this master mix and 4.8µl of standard/sample were added to each strip mini tube. A negative control reaction containing master mix, primers and nuclease free water was also included in each run to identify any contamination of reagents or non-specific amplification. Tubes were then added to the Qiagen Rotor-Gene-Q qPCR machine which pre-incubated at 95°C for 10 minutes in order to activate the hot start polymerase in the master mix, followed by a 45 cycle run (95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds) to amplify target cDNA, followed by ramp from 72°C to 95°C melt curve to examine primer specificity. All samples analysed for genes of interest were normalised against the levels of at least one references gene from: B2M, ESP8, and P4HB.

### 2.5.7. IL-6r RT-PCR

RT-PCR was used to semi-quantitatively analyse the levels of the full length (219bp) and the cleaved isoform (125bp) of IL-6r mRNA in fibroblast cultures using previously published primer sequences that amplify across the spliced intron (Vermes *et al.*, 2002). cDNA from fibroblast cultures was used for a PCR reaction in the GeneAmp® PCR System 9700 thermocycler. Reactions were run in 8-tube PCR strip tubes and included a negative control tube (water instead of cDNA) and a positive control tube (THP-1 cDNA). Each reaction contained 12.5µl 2x GoTaq® Green Master Mix (Promega), 2µl 10µM forward/reverse primer mix, 2.5µl cDNA and 8µl nuclease free water. Each sample was analysed using IL-6r primers and ESP8 primers as a reference gene.

The cycling conditions were: an initial incubation at 94°C to activate the polymerase, before a cycle of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds (repeated for 30 cycles), and a final incubation at 72°C for 5 minutes before cooling to 4°C.

The PCR product was assessed by electrophoresis on a 1.5% agarose gel (1.5g agarose in 100ml 1X TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA) with 10µl of Diamond™ Nucleic Acid Dye. The molten agarose was mixed thoroughly by swirling and then poured into a gel mould containing well comb/s. Gels were left to set for ~30 minutes before the removal of the well combs and placement into the tank chamber. 1X TAE buffer was added to the tank chamber to cover the gel by ~5mm.

10µl of each PCR reaction was loaded per gel lane, alongside one lane containing 5µl of Quick-Load® Purple Low Molecular Weight DNA Ladder (New England BioLabs) for the estimation of PCR band sizes. Gel electrophoresis was generally conducted at 100V for between 1 and 2 hours to separate bands enough to identify the 219bp and 125bp bands. Gels were imaged using the SYBR-Green protocol for Prominent Bands on the BioRad Gel Doc+ system.

Band sizes of the fragments of interest and the reference gene were quantified in each sample using the BioRad ImageLab 3.1 software, allowing normalisation and semi-quantitative estimation of relative mRNA expression.

# Chapter 3: Characterisation of BET protein expression in keloid scars

## 3.1. Introduction

Whilst the make-up of keloids is recognised to be distinct from normal tissue, this is not well characterised in current literature and many conflicting findings are reported. Furthermore, the differences between dermal fibroblasts isolated from normal skin and keloid scar, and whether these differences are retained *in vitro*, are not fully described. There is evidence in the literature for epigenetic changes in keloid fibroblasts including altered histone methylation and acetylation (Russell *et al.*, 2010) and overexpression of acetylation modulating enzymes (Fitzgerald O'Connor *et al.*, 2012) which is likely to cause transcriptional and behavioural changes. This project investigated the role of a family of epigenetic regulator proteins called BETs, which interact with acetylated lysines predominantly on histone proteins to modulate transcription factor and transcriptional machinery binding as was introduced previously.

The BET protein family contains four members: BRD2, BRD3, BRD4 and BRDT, but BRDT is exclusively expressed in the testis (Matzuk *et al.*, 2012). BRD2, BRD3 and BRD4 are ubiquitously expressed nuclear proteins produced by a wide range of cell types including those in the skin (Stonestrom *et al.*, 2015). Information reported by The Human Protein Atlas describes expression, as determined by RNA seq, in a range of cells in the skin, predominantly in keratinocytes, although IHC data does not always confirm protein expression. However, there is information from the literature that does suggest the presence of BET proteins in dermal fibroblasts (**Table 3-1**) (Ijaz *et al.*, 2017).

***Table 3.1* - Summary of BET expression data from Human Protein Atlas.**

| Member | Protein (IHC) | Transcription (RNA-seq) |
|---|---|---|
| **BRD2** | Not detected in any cells (tested two antibodies) | Expression in a range of cells (55-60% keratinocytes, 40-45% other cells) |
| **BRD3** | Medium positivity in fibroblasts, keratinocytes, Langerhans. High positivity in melanocytes. | Expression in a range of cells (55-60% keratinocytes, 40-45% other cells) |
| **BRD4** | Heterogeneous results, some samples show high expression in fibroblasts, keratinocytes, Langerhans and melanocytes. | Expression in a range of cells (55-60% keratinocytes, 40-45% other cells) |

Although BET proteins appear to have an important role in normal development and homeostasis (Houzelstein *et al.*, 2002; Belkina *et al.*, 2014; Stonestrom *et al.*, 2016), their overexpression and/or differential activity has been associated with a variety of diseases including fibrosis in various organs. For example, BRD4 is highly enriched at fibrosis-associated enhancer regions in human stellate cells, suggesting that BRD may play a role in regulating liver fibrosis (Ding *et al.*, 2015). The binding of BRD4 was also found to be associated with a number of profibrotic transcription factors such as SRF (serum response factor), SMAD3 and NF-κB (Ding *et al.*, 2015). These are transcription factors that also appear in keloid literature (Messadi *et al.*, 2004; Phan *et al.*, 2005; Velasquez *et al.*, 2013) and therefore may indicate some role for BRD4 in keloid pathology. Recently it has even been shown that TGF-β1 treatment of dermal fibroblasts induces BRD4 interaction with SMAD3 which can promote myofibroblast differentiation (Ijaz *et al.*, 2017).

Increased Brd4 expression was also seen by IHC in experimentally obstructed kidneys (which would go on to become fibrotic) and also in tissue from patients suffering fibrotic kidney conditions (Xiong *et al.*, 2016). Both the liver and the kidney fibrosis papers acknowledge that the beneficial effect of BET family inhibition in disease models they observe may not be exclusively due to inhibition of BRD4, and that BRD2 and/or BRD3 may also have important roles, although they do not explore this further. Interestingly, the Ijaz study noted that BRD4 suppression could not suppress all myofibroblast associated transcription, unlike BET inhibition, which indicates a role for BRD2 also (Ijaz *et al.*, 2017).

Another study showed that both BRD2 and BRD4 were highly important in regulating the fibrotic response of human lung fibroblasts to growth factor stimulation and again that pharmacological inhibition was beneficial by diminishing bleomycin-induced lung fibrosis in mice (Tang *et al.*, 2013a). Therefore, this project aimed to have a broad approach by

investigating BRD2, BRD3, and BRD4 in keloid pathology rather than focussing on one member of the family.

Preliminary data from our lab suggested that BRD2 and BRD4 may be overexpressed in keloid tissue by IHC and here the aim was to further investigate the expression of BET proteins in cells and tissue in normal skin and keloid scars with the hypothesis that some or all BET protein members may be upregulated in keloid tissue and this may contribute to disease.

The main objectives were:

1. To better characterise the fibrotic environment of keloid tissue.
2. To investigate BET expression in normal and keloid tissue with particular focus on the dermal compartment.
3. To investigate BET expression specifically in dermal fibroblasts from normal skin and keloid tissue.

## 3.2. Results

### 3.2.1. Keloid patient information

Firstly, to understand the demographic details of the keloid patients in our study, data from the Patient Information Sheets (**Figure 7.1**) from consented patients were assimilated. In total 27 keloid patient information sheets were collected.

It was found that more keloid patients in our study were female (24/37), with the majority of patients being under the age of 40 (**Figure 3.1A**). The mean duration of keloid scars was 5.95 years and the median duration was 5 years, although the majority of keloid scars were less than 5 years old, with one patient estimating that their keloid was over 15 years old (**Figure 3.1B**). Over 67% (25/37) of patients had more than one keloid scar (**Figure 3.1C**) and over 40% (15/37) of patients had some family history of keloid formation (**Figure 3.1D**). Seven patients reported that their keloid scars were itchy and seven additional patients reported that their keloid scars were both itchy and painful. Interestingly, no patients reported pain in the absence of itchiness (**Figure 3.1E**).

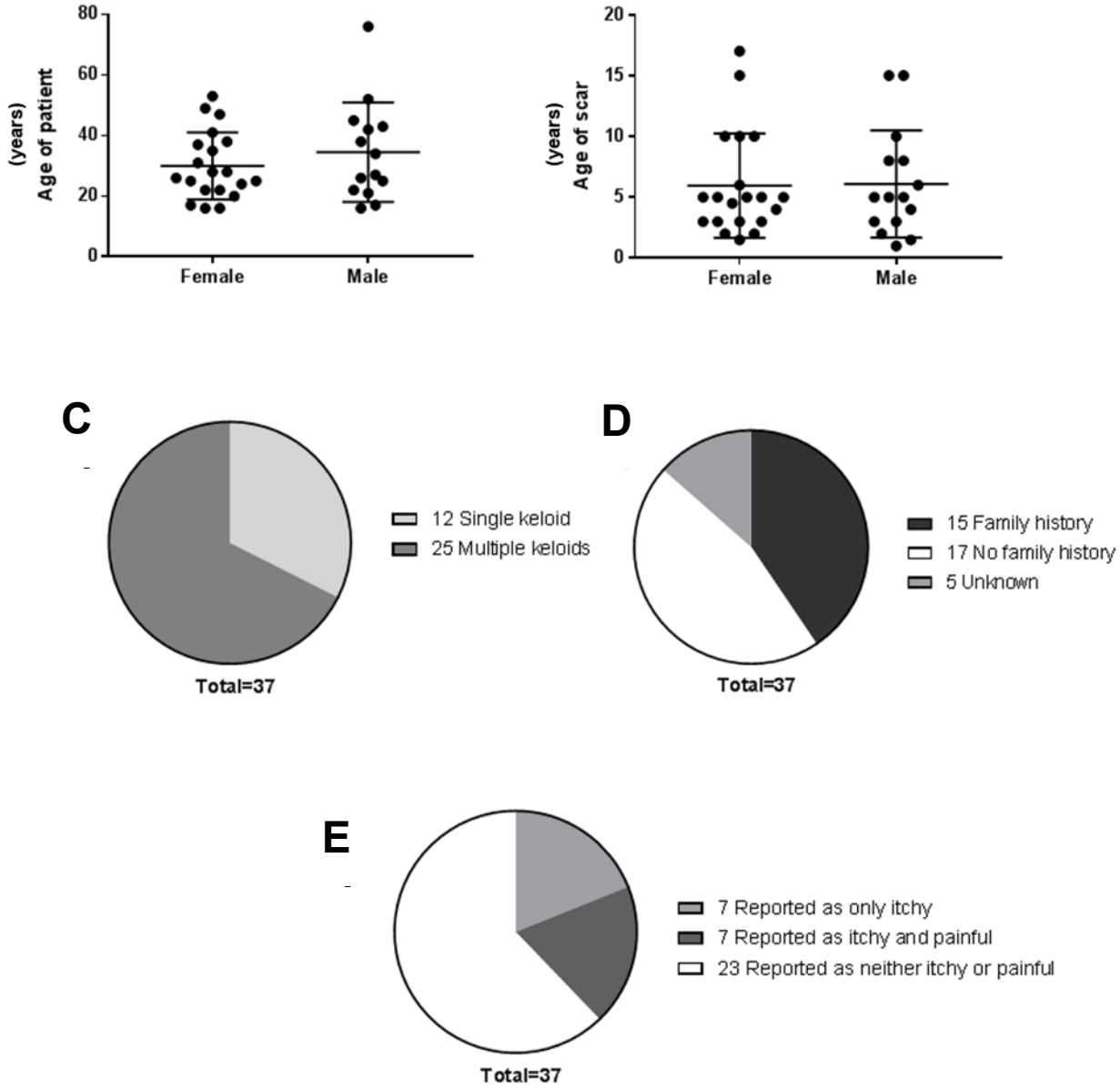



***Figure 3.1*** **- Keloid patient information based on completed information sheets.** (**A**) Sex and age of patients, mean +/- SEM. (**B**) Sex and age of keloid scar, mean +/- SEM. (**C**) Proportion of patients with a single or multiple keloids. (**D**) Proportion of patients with a family history of keloids. (**E**) Proportion of patients with itchy or painful keloids.

### 3.2.2. Fibrotic environment of keloid tissue

In order to visualise first-hand the well documented and grossly obvious fibrotic reaction in keloids, the matrix appearance of normal skin, normal scar and keloid scars was examined histologically with Haematoxylin Van Gieson's staining. Van Gieson's stain was developed in 1889 as a staining method to visualise connective tissue, with collagens staining in red/pink and cytoplasm in yellow (Segnani *et al.*, 2015). Staining was conducted by Carl Hobbs (Wolfson Centre for Age Related Disease, King's College London) on formalin fixed, paraffin embedded sections from three normal skin, two normal scar and two keloid scar patient samples. Stained slides were imaged using the Hamamatsu NanoZoomer-XR digital slide scanner. Image J was used to analyse the amount of 'white space' in a field of view within the dermis, avoiding dermal appendages and damage to tissue, using colour thresholding. White space was measured to assess the loss of basket weave patterning in normal skin and to attempt to quantify the qualitative differences seen between normal scar and keloid scar dermal staining. For each sample, 3 independent fields of view with the x20 objective were randomly selected within 500µm of the epidermal layer whilst avoiding appendages.

There was an increase in the intensity of staining with a denser distribution of staining in scar tissue (both normal and keloid) compared to normal skin (**Figure 3.2**). Moreover, there was more white space in normal skin, indicative of the classic basket-weave pattern of collagen that gives skin tensile strength and flexibility (McGrath, Eady and Pope, 2004) . Interestingly, the keloid scar samples examined appeared to have slightly more white space than normal scar, suggesting that the arrangement of collagen fibres may be different. Upon examining keloid dermis at a greater magnification, a patchwork pattern became apparent, with dense pink collagen areas and regions of thick collagen fibres along the sectioning plane (**Figure 3.2**).

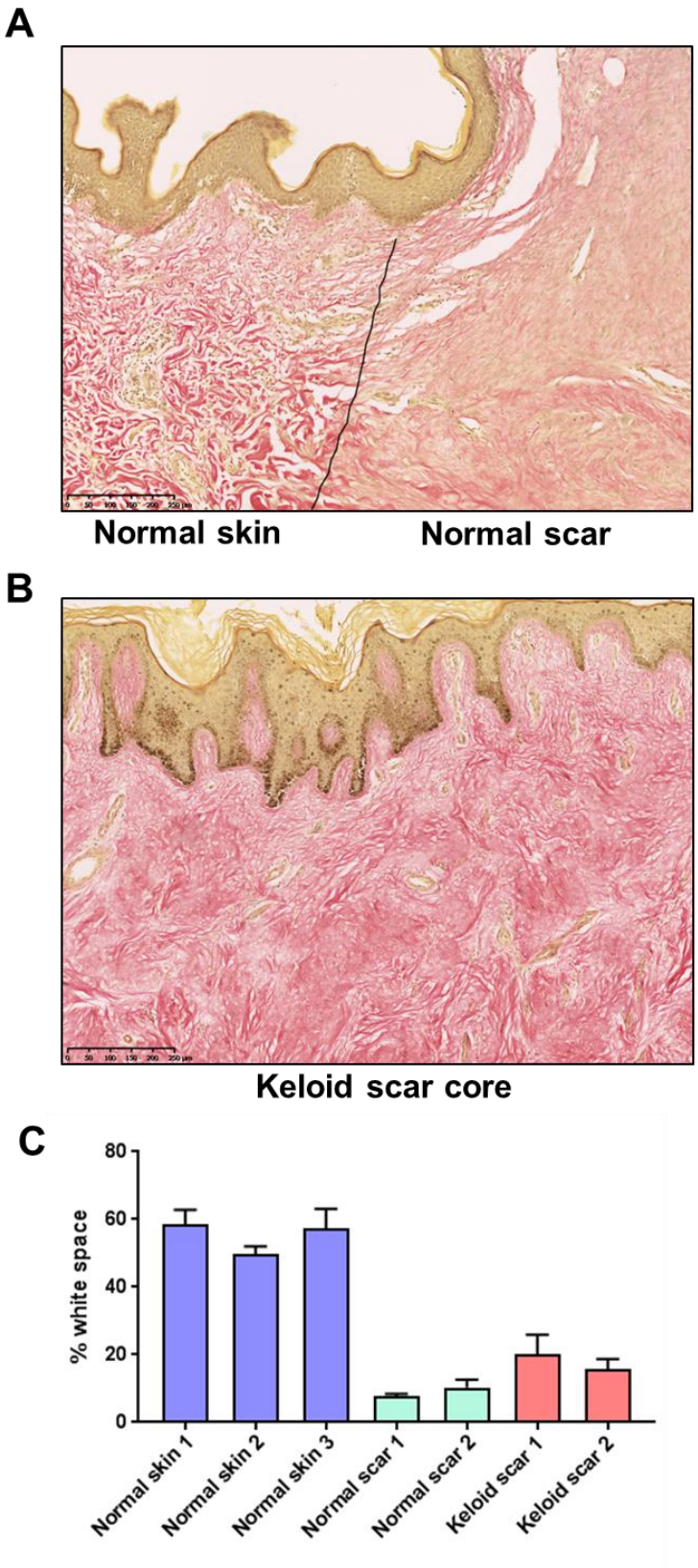



***Figure 3.2* - Haematoxylin Van Gieson (HVG) staining of dermal tissue.** HVG was conducted on formalin fixed, paraffin embedded tissue from 3 normal skin, 2 normal scar and 2 keloid scar samples, scale bar = 250µm. (**A**) Staining in a representative normal scar sample (NS) with a normal skin border (left hand side of the image), (**B**) Staining in the dermis of a representative keloid scar sample (KS). (**C**) Quantification of 'white space' within the dermis of HVG stained samples, n= 3 technical replicates where each replicate is an independent field of view with x20 objective.

To further explore the production of factors important to the formation of this fibrotic matrix, ECM transcripts were investigated by RT-qPCR.

RNA was extracted from 12 samples in total (**Table 3.2**). These samples were collected during a targeted collection period of ~4 months from local sources of tissue i.e. Cancer Centre keloid clinic at Guys, in order to minimise sample processing time and therefore increase RNA integrity. Homogenisation of the tissue, even with the TissueLyser, was difficult and therefore yield was lower than expected. However, RINs were better than previously achieved; normal skin all showed high integrity RNA that would even be suitable for RNAseq analysis (i.e. ≥ 7-8). Keloid RNA integrity was more variable with RINs ranging from 1 to 8.

A 200ng reverse transcription reaction was conducted on 10 of the 12 samples, excluding the two samples that had an undetectable RIN or extremely low RIN (1). Relative expression for each primer pair was calculated from extrapolation to a standard curve, and normalisation to the reference gene P4HB. Keloid tissue had significantly higher levels of fibronectin (FN), collagen 1a1 (COL1A1) and collagen 3a1 (COL3A1) compared to normal skin and significantly lower levels of αSMA (ACTA2) (**Figure 3.3**).

***Table 3.2* - Summary of tissue RNA extractions.**

| Type of tissue | Sample ID | RIN | Conc (ng/µl) |
|---|---|---|---|
| **Normal Skin** | N160517 | 8.9 | 50 |
| **Normal Skin** | N130317 | 8.2 | 46 |
| **Normal Skin** | N150517 | 7.9 | 59 |
| **Normal Skin** | N040317 | 7.5 | 28 |
| **Normal Skin** | N070317 | Undetectable | <1 |
| **Adjacent keloid skin** | KN130317* | 8.4 | 35 |
| **Keloid scar** | K130317* | 6.1 | 98 |
| **Keloid scar** | K080217 | 1 | 11 |
| **Keloid scar** | K200217 | 4.6 | 47 |
| **Keloid scar** | K060317 | 4.4 | 101 |
| **Keloid scar** | K230317A** | 8.4 | 130 |
| **Keloid scar** | K230317B** | 6.6 | 21 |

** = matched keloid and adjacent skin, **= same patient with two keloids on left and right ear lobes. Blue samples are those used in all qPCR analysis, orange samples are additional samples used in BET mRNA analysis (3.2.7.).*

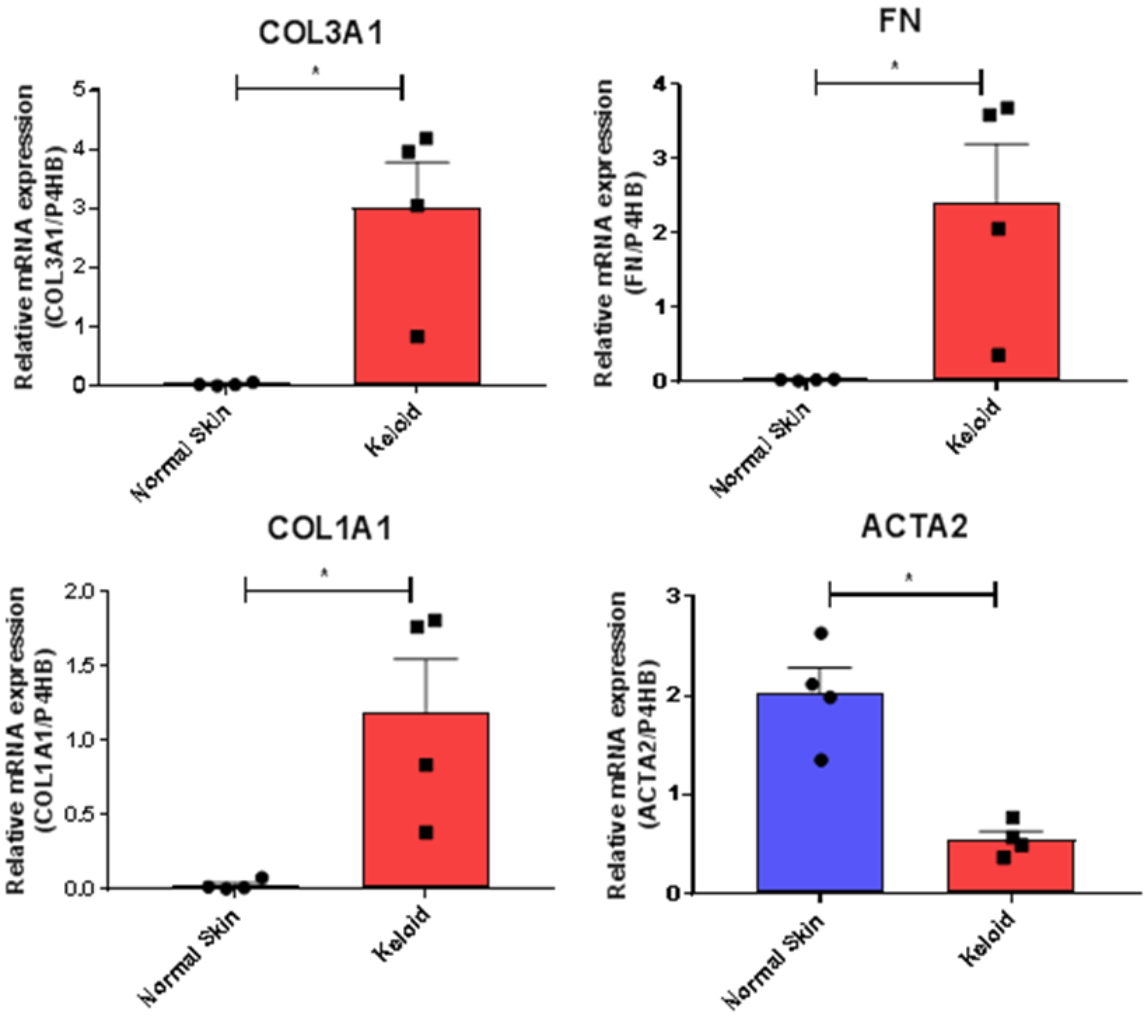



***Figure 3.3*** **- RT-qPCR for fibrosis associated genes in normal skin and keloid tissue.** RT-qPCR was conducted on 4 normal skin and keloid scar tissue samples which were normalised to the reference gene P4HB. All four transcripts were quantified based on standard curve and showed a statistically significant difference (*$p<0.05$) by a non-parametric, unpaired testing (Mann-Whitney). 4 biological replicates in technical duplicate, n=4, mean +/- SEM.

### 3.2.3. Expression of BET mRNA in tissue

mRNA extracted from normal skin and keloid scar tissue was then used to examine the expression of BET proteins, BRD2, BRD3 and BRD4 (**Figure 3.4**). A subset of keloid tissue had higher BRD2 expression than normal skin. Interestingly, the two keloids that showed similar levels to normal skin were two samples from the same patient. Normal skin and keloid scar appeared to have similar levels of BRD3. Normal skin had a slightly higher amount of BRD4. No significant difference was found between normal skin and keloid scar in the expression of any of the BET proteins, as analysed by Mann Whitney test (BRD2 $p=0.1506$, BRD3 $p>0.99$, BRD4 $p= 0.222$).

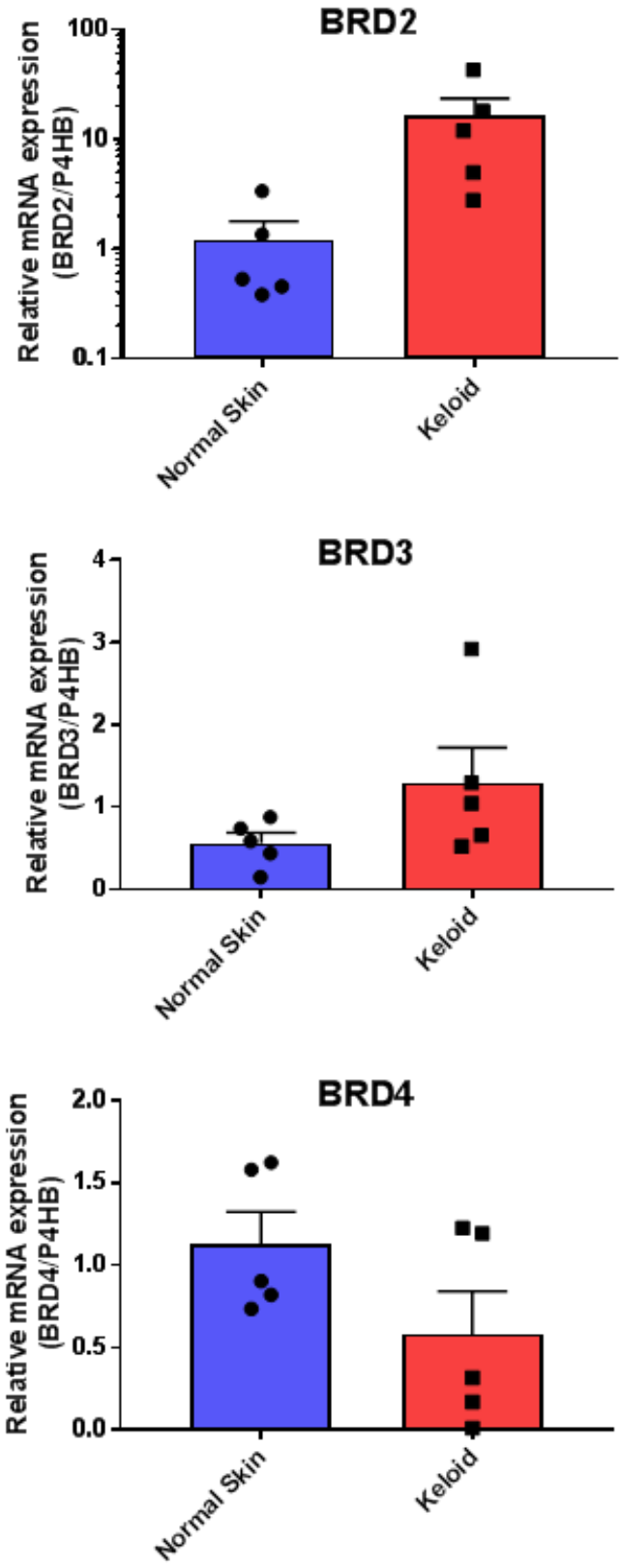



***Figure 3.4*** **- mRNA expression of BRD2, BRD3 and BRD4 in tissue.** qPCR was conducted on 5 normal skin and keloid scar tissue samples which were normalised to the reference gene P4HB. All four transcripts were quantified based on standard curve. 5 biological replicates in technical duplicate, n=5, mean +/-SEM.

### 3.2.4. BRD2, BRD3 and BRD4 protein expression in tissue

As there was some indication that there may be differences in BRD2 and BRD4 mRNA expression, further analysis was conducted to quantify and localise the proteins. In order to focus on the dermis of the tissue, which is where the majority of the pathology of keloid unfolds, de-epithelised tissue from both keloid scars and normal skin was homogenised and protein was extracted and quantified. Western blots were then run with equal quantity of protein from n=4-6 keloid scar dermis and normal skin dermis and probed for BRD2, BRD3, BRD4 (rabbit polyclonal antibodies, GSK) and GAPDH for a loading control.

A low basal expression of BRD2 was seen in all patient samples, with overexpression in a number of keloid samples. Interestingly, where two keloids from the same patient were examined (K1 and K2, and K4 and K5), there was overexpression in both patients (**Figure 3.5**). BRD3 results showed relatively consistent expression across all patient samples, except for a single keloid sample (K4) which had approximately double the amount of BRD3. This keloid sample was also the highest expresser of BRD2. Mirroring what was seen at the mRNA level, BRD4 expression seemed to be higher in normal skin (especially samples 3-6) (**Figure 3.5**).

The banding patterns of the BET proteins were not the size expected based on Ensembl.org size predictions, with all BET protein bands between ~60-70kDa, whereas full-length BRD2 was expected to be ~88kDa, BRD3 to be ~80kDa and BRD4 to be ~155kDa (protein sequence to molecular weight prediction from http://www.signaling-gateway.org).

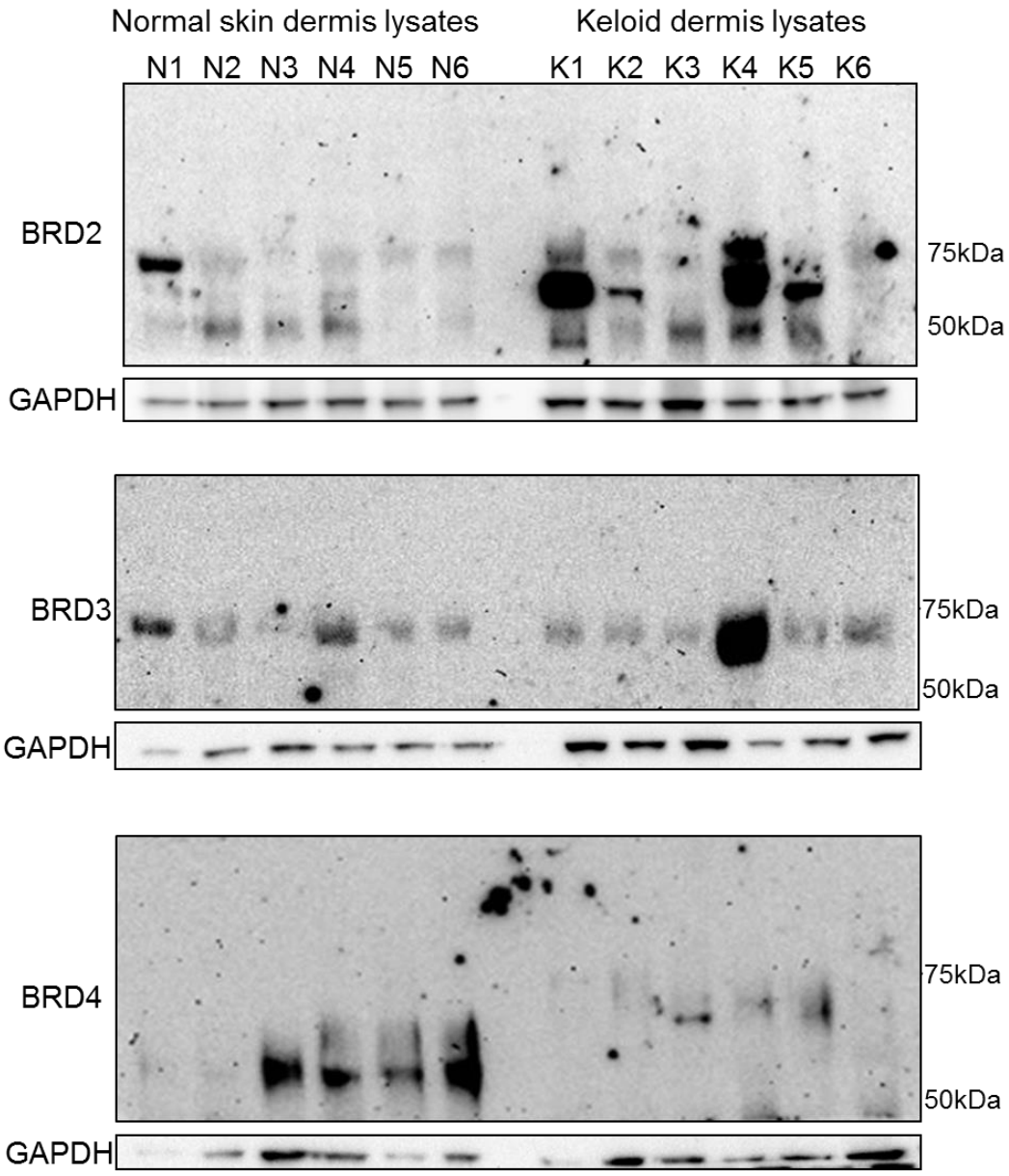



***Figure 3.5*** **- Expression of BET proteins in dermal tissue lysate.** Western blot of normal skin and keloid scar dermal tissue lysates probed for BRD2, BRD3 and BRD4, and GAPDH as a loading control, n=4*-6. *(K1/K2 and K4/K5 are different keloids from the same patient).

### 3.2.5. BET expression in a range of cell types

Due to the apparent overexpression of BRD2 protein in several keloid dermal lysates with an unexpected molecular weight, a range of cell types were analysed by western blot to examine antibody banding patterns for BRD2. As the purpose of this experiment was to qualitatively examine BET expression in these cells types, protein lysates were not quantified prior to loading.

BETs were detected in all the cells types tested but with differential banding (**Figure 3.6**), which is summarised in **Table 3.3**.

***Table 3.3* - Summary of GSK BRD2 antibody banding in a range of cell types.**

| Cell name | Cell origin | Species | Brd banding (~kDa) |
|---|---|---|---|
| **NDF** | Primary dermal fibroblasts | Human | 60, 65 |
| **KERA308** | Keratinocyte | Mouse | 90, 120, 160 |
| **HUVEC** | Umbilical vein endothelial cells | Human | 65, 120, 140 |
| **SK-OV-3** | Ovarian cancer | Human | 120, >250 |
| **MDCK** | Kidney | Canine | 40, 45, 120, >260 |
| **HMRSV5** | Peritoneal mesothelial cells | Human | No bands detected |
| **RAW264.7** | Macrophage | Murine | <37, 40, 45 |
| **HEK-293** | Embryonic kidney cells | Human | Smear from <37 to 42 |
| **THP-1** | Monocyte | Human | 40, 100, 120 |

*Approximate banding sizes were estimated by western blot as compared to the Precision Plus Protein™ Dual Color Standards protein ladder #1610374.*

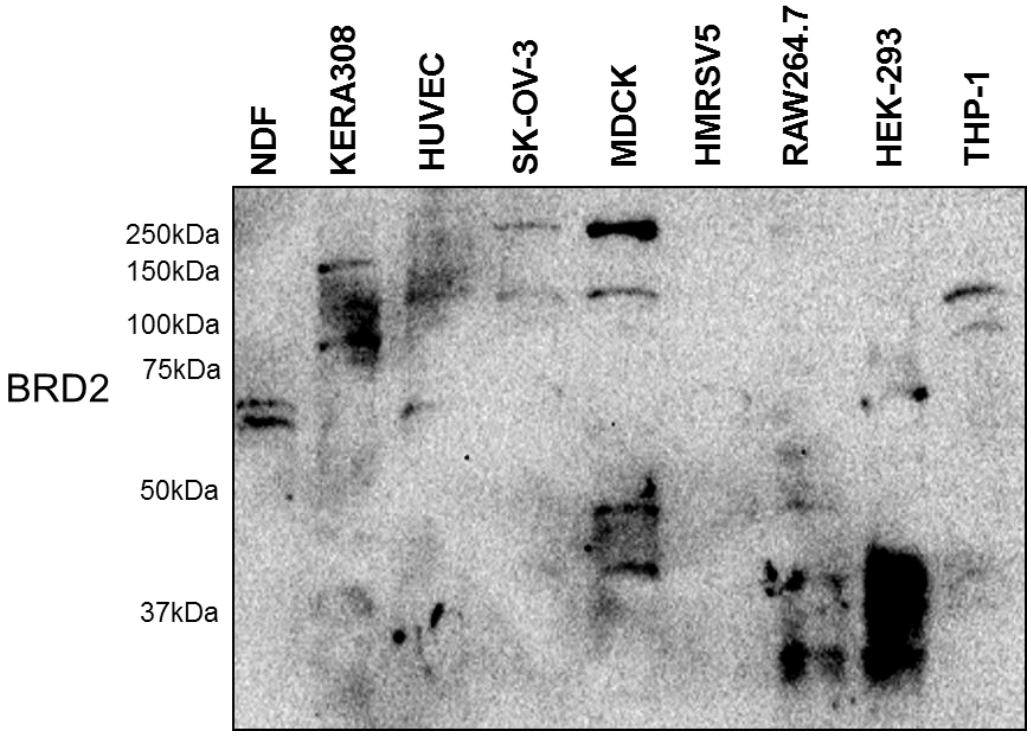



***Figure 3.6* - Western blot showing BRD2 protein expression patterns in a range of cell lysates.** Lysates were created from a range of cell types to evaluate specificity of the BRD2 antibody generated at GSK.

### 3.2.6. Protein expression pattern of BRD2 and BRD4 in tissue

The westerns showed BET expression in the dermis, but with our particular interest in expression by dermal fibroblasts, the localisation of BET proteins were investigated by immunohistochemistry (IHC). DAB-IHC staining for BRD2, BRD3 and BRD4 was carried out using the Ventana automated staining system. This system offered a highly controlled staining process increasing the ability for comparison between tissue types.

Initially this staining was intended to be analysed quantitatively in the dermis using Definiens image analysis software, however there was extensive damage to tissue sections, particularly during antigen retrieval, which was found to be necessary for optimal staining. Unfortunately, despite testing a range of BRD3 antibodies including commercially available and the polyclonal antibody used for western blots, there was no adequate IHC staining.

In contrast to what is currently reported by the Human Protein Atlas (**Table 3.1*)***, many cells in the dermis tested appeared to show moderate to high positivity for BRD2. Immune cells were generally positive for both BRD2 and BRD4 (data not shown). Fibroblasts, indicated by their distinctive elongated nuclear shape, appeared to be almost all positive for BRD2 in keloid scar tissue, whereas they showed lower levels of staining in normal scar and variable staining in normal skin (**Figure 3.7**). The staining pattern of BRD2 also indicated the possibility for presence of this protein in the cytoplasm. BRD4 expression was strong in normal skin, normal scar and keloid scar, with clear nuclear localisation (**Figure 3.8**).

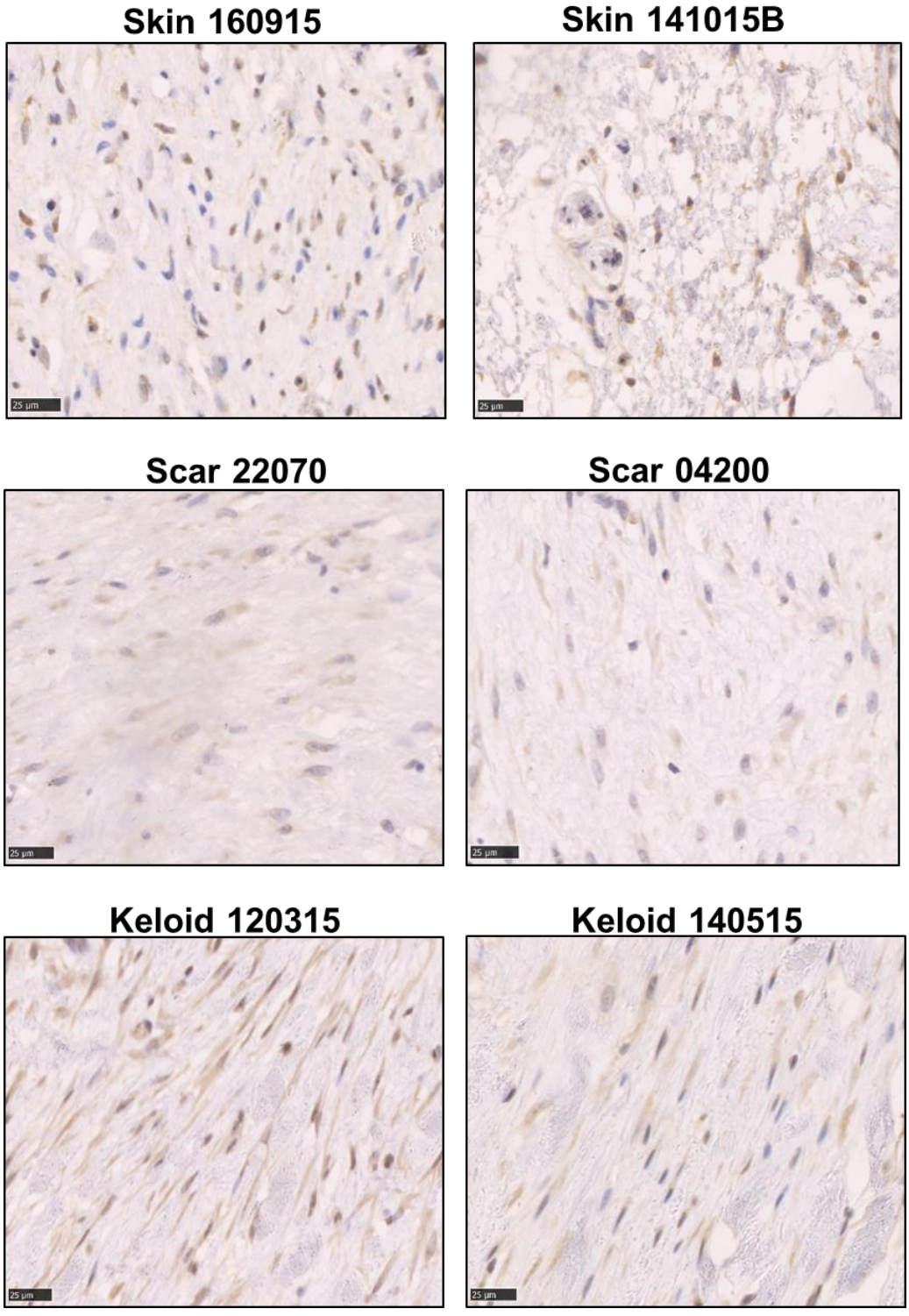



***Figure 3.7*** **- BRD2 expression in normal skin, normal scar and keloid scar dermis.** Two representative images for each tissue type demonstrating the expression pattern of BRD2 following DAB-IHC using the Ventana staining system. Brown staining denotes BRD2 expression, blue staining shows nuclei by haematoxylin staining. Scale bar = 25µm.

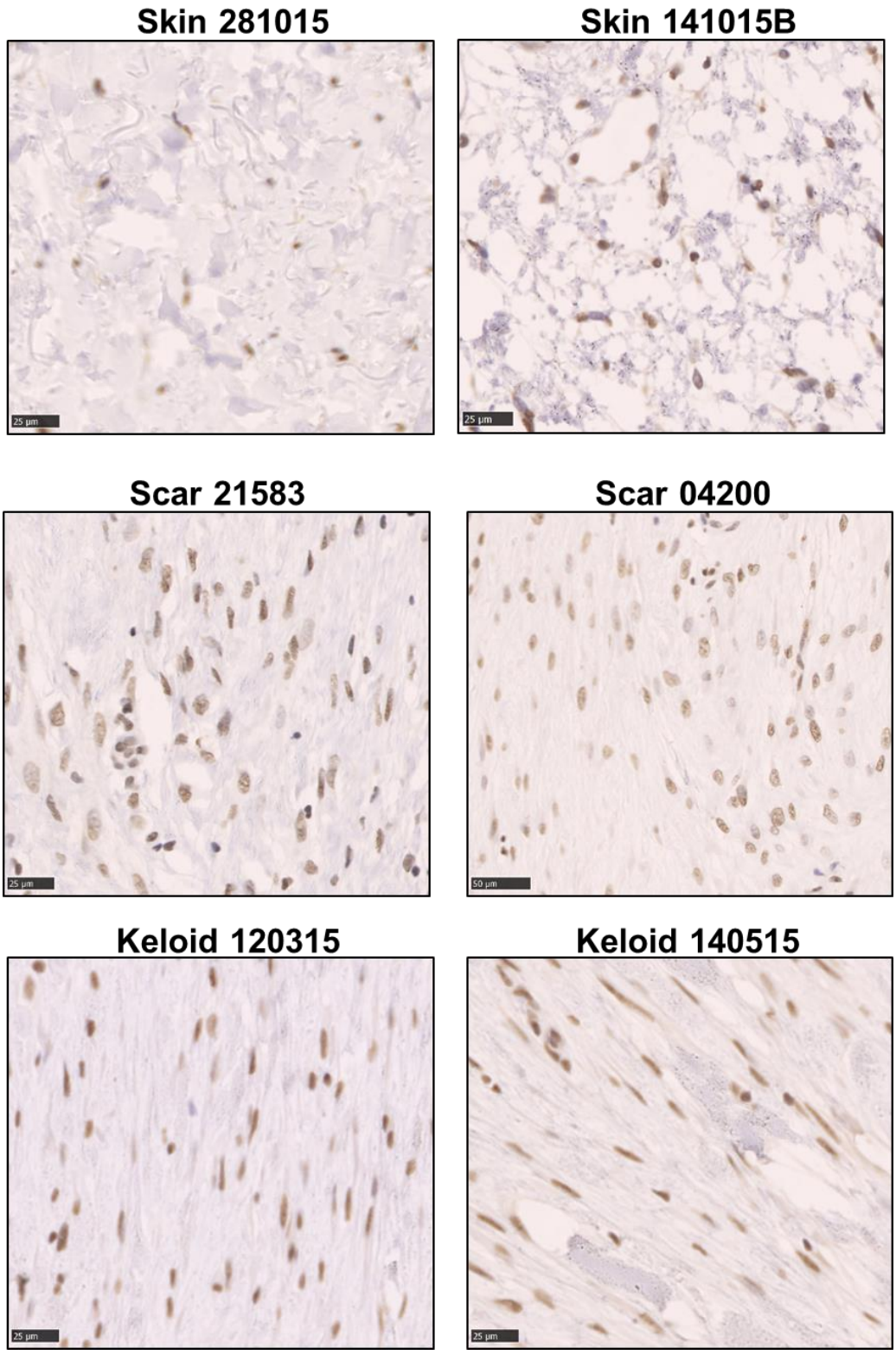



***Figure 3.8* - BRD4 expression in normal skin, normal scar and keloid scar dermis.** Two representative images for each tissue type demonstrating the expression pattern of BRD4 following DAB-IHC using the Ventana staining system. Brown staining denotes BRD4 expression, blue staining shows nuclei by haematoxylin staining. Scale bar = 25µm.

### 3.2.7. BRD2 and BRD4 expression in cultured fibroblasts

Following the indication from the IHC experiments that fibroblasts expressed moderate to high levels of BRD2 with a possible increase in expression in keloid fibroblasts, protein and RNA was isolated from normal dermal fibroblast (NDF) and keloid dermal fibroblast (KDF) cultures to examine BRD2 expression *in vitro*. Cells were also fixed in 4% PFA on poly-L-lysine coated coverslips for immunofluorescent staining to investigate cellular localisation of BRD2 protein.

Firstly, western blots were run for 6 NDF and 6 KDF protein isolations, probing with the rabbit polyclonal BRD2 antibody from GSK used in **Figure 3.5**. This showed equal protein expression of BRD2 in these cultures (**Figure 3.9A**). Immunofluorescent staining also showed similar staining intensity between the NDF and KDF tested, with nuclear staining as expected, but again some indication of cytoplasmic localisation of the protein (**Figure 3.9B**).

NDF and KDF mRNA was also analysed for all the BET family members by qPCR, with results normalised to the reference gene B2M. Primers for BRD2, BRD3 and the short and long isoforms of BRD4 (BRD4S and BRD4L respectively) were used. This also showed no marked difference in expression between NDFs and KDFs (**Figure 3.10A**), including no difference in the ratio of BRD4S to BRD4L. There was medium to strong correlation of all BET members (**Figure 3.10B**).

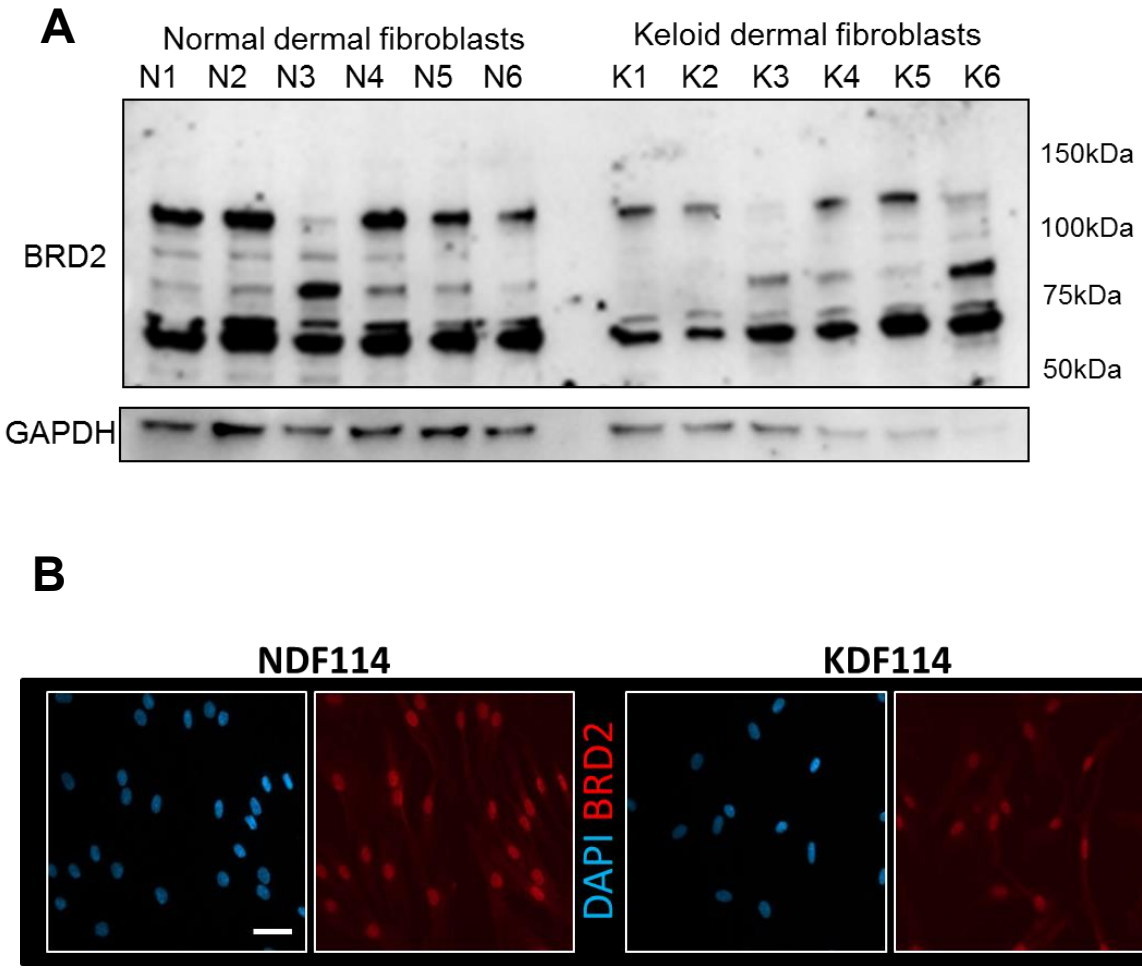
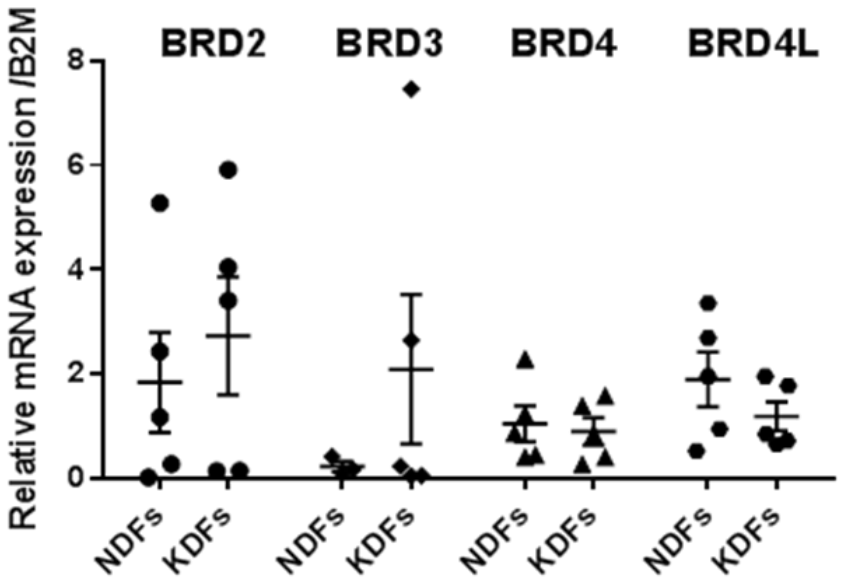



***Figure 3.9* - Expression of BRD2 proteins in cultured dermal fibroblasts.** (**A**) Western blot of cultured NDF and KDF lysates probed for BRD2 and GAPDH as a loading control, n=6. (**B**) Immunofluorescence BRD2 staining of cultured NDF114 (keloid patient matched 'normal' dermal fibroblast) and KDF114, formalin-fixed onto coverslips.

**A**




**B**

Spearman's R

| | BRD2 | BRD3 | BRD4 | BRD4L |
|---|---|---|---|---|
| BRD2 | | 0.54 | 0.39 | 0.62 |
| BRD3 | 0.54 | | 0.62 | 0.67 |
| BRD4 | 0.39 | 0.62 | | 0.86 |
| BRD4L | 0.62 | 0.67 | 0.86 | |

***Figure 3.10* - BET mRNA expression in NDFs and KDFs.** (**A**) Relative expression of BRD2, BRD3, BRD4 and BRD4L were normalised to the reference genes B2M. All four transcripts were quantified based on standard curve. 5 biological replicates in technical duplicate, n=5, mean +/-SEM. (**B**) Spearman's correlation (non-parametric) analysis of the BET member mRNA transcripts.

### 3.2.8. Expression of BRD2 protein following stimulation of cultured fibroblasts

As BRD2 has been shown to potentially be expressed in fibroblast-like cells by IHC and within keloid dermal tissue, to examine the regulation of BRD2 fibroblast cultures were treated with a range of cytokines and growth factors to examine whether this might be able to induce expression in fibroblasts. An NDF line was treated for 72 hours with 10ng/ml IL-6, 10ng/ml IL-17A, 10ng/ml TGF-β1 and 20ng/ml PDGF-BB and protein was harvested for western blot analysis. These factors were chosen as they have all been to linked to keloid pathology. It was found that the keloid associated factors may induce a small increase in BRD2 expression (**Figure 3.11A)**.

Lysates from NDFs treated with other inflammatory type factors were generated by Shuhanaz Begum (MSc student) were also used for western blot analysis of BRD2 expression and it was found that lipopolysaccharide (LPS) and tumour necrosis factor alpha (TNFα) were capable of increasing levels of BRD2 in two NDF lines (one representative blot shown) (**Figure 3.11B)**.

Lastly, lysates from an NDF and KDF line were treated for 24 hours with conditioned media taken from tissue *ex vivo* models and diluted 1:3 (due to the limited volume of conditioned media available (**Figure 3.11C**). Culture with normal skin *ex vivo* conditioned media (N-CM) appeared to have no effect on BRD2 protein expression in the NDF line, but culture with conditioned media from keloid tissue *ex vivo* cultures (K-CM) caused a decrease in BRD2 protein expression in the KDF line.

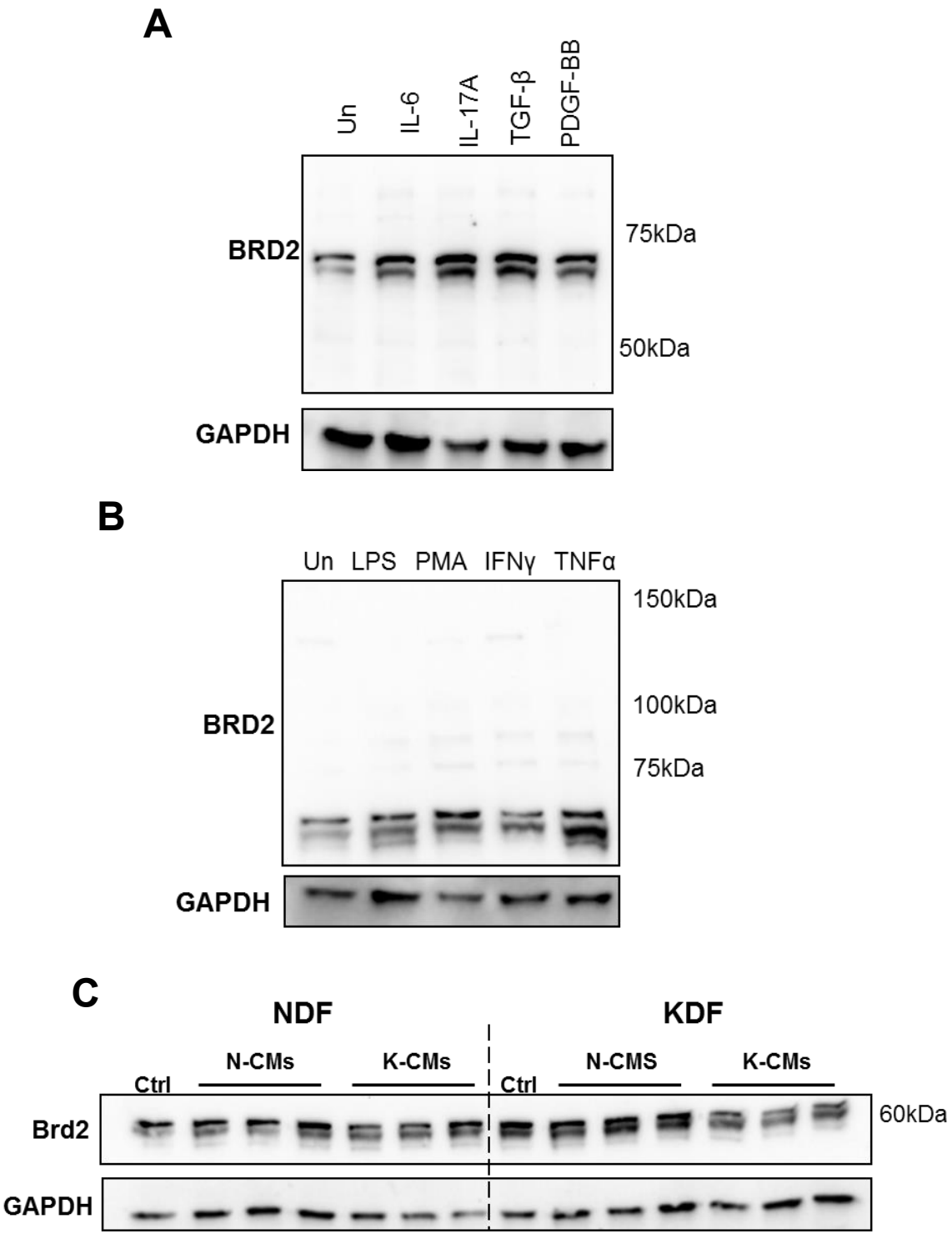



***Figure 3.11*** **- Expression of BRD2 in cultured fibroblasts after stimulation.** (**A**) Western blot of cultured NDF lysates after 72 hour stimulation with 10ng/ml IL-6, 10ng/ml IL-17A, 10ng/ml TGF-β1 and 20ng/ml PDGF-BB. (**B**) Western blot of cultured NDF lysates after 72 hour stimulation with 0.05µg/ml LPS, 10ng/ml PMA, 5ng/ml IFNγ, 100ng/ml TNFα. (**C**) Western blot of cultured NDF and KDF lysates after 24 hour stimulation with 1:3 diluted *ex vivo* culture conditioned media from 3 different patients. All blots were probed for BRD2 and GAPDH as a loading control.

## 3.3. Discussion

In this study, we have confirmed literature reports that COLA1, COL3A1 and FN are increased in keloid tissue compared to normal skin, we have also confirmed a difference in collagen distribution in keloid tissue compared to both normal skin and normal scar. We have also contributed new knowledge in that Brd2 is expressed both in dermal fibroblasts and in whole skin tissue. We have novel data that suggests a possible overexpression of Brd2 in keloid scar dermis with a potential differential isoform of 60kDa that has not been reported in the literature and could have altered function.

During the span of this project, information for 37 patients was collected when informed consent was given for sample collection. This information was analysed to better understand the patient demographics and characteristics of our clinical samples. Firstly, analysis of the patient information sheets showed that around two-thirds of patients had multiple keloid scars. A much bigger cohort of patients (211) in an Afro-Caribbean population analysed by Bayat *et al* in 2004 found that 42% of their patients had keloids in multiple anatomical sites and also found that this did not vary between males and females (Bayat *et al.*, 2004). However, it is unclear whether patients in our study who stated that they suffered from multiple keloid scars had multiple anatomical sites that were affected. Since many keloid scars are located on the earlobe as a result of piercing, it is likely that both ears are affected and could be classified as multiple scars and account for some over-reporting of multiple keloid prevalence. There is not yet information in the literature regarding the cause of multiple keloids, however the Bayat study did show that in their cohort of patients, there is a greater incidence of multiple keloids in younger patients, females and those with a family history of keloids (Bayat *et al.*, 2004).

The Bayat study also found that between 41 and 54% of patients reported a family history which is comparable to our results where 15 of 33 patients had confirmed family history. Despite a definite familial link for keloid risk, there has been a number of studies to elucidate the genetic basis of keloid formation with no consistent genes or pathways proven to be causative across the multiple populations studied.

Additionally, the Bayat study reported that 35% of their patients were male and 65% were female, which again was similar to our findings that 24 out of 37 patients were female (64.8%). It is possible that due to the cosmetic nature of many keloid scars, females are more likely to seek care especially as it is often reported that keloid scars have equal rates of occurrence in males and females, (Gauglitz *et al.*, 2011). However, there is good evidence to suggest that there is a true biological bias towards keloid scars occurring in females, including the growth and recurrence of keloids during pregnancy and decrease or absence of keloid formation in menopause, which has been reported for many decades and indicates a role for female hormones

although is limited to clinical observations (Moustafa and Abdel-Fattah, 1975; Park and Chang, 2012; Kim *et al.*, 2013).

Whilst 17 out of 37 patients in our study reported their scars were itchy and another 17 reported their scars as itchy and painful, there were no patients that reported pain in the absence of itching. Pruritus is a significant clinical issue in keloid scars and is therefore a focus for treatment strategies (Bock *et al.*, 2006). Inflammation and itch may also be directly linked through the influence of inflammatory mediators such as histamine by mast cells, which have been reported to be present in keloid scar tissue (Bagabir *et al*. 2012a). Additionally, pruritus and temperature sensitivity in keloids was linked to abnormalities in small nerve fibre functions which were postulated to be caused by the dense collagenous matrix that may compress or damage nerves (Lee *et al*. 2004).

The dense collagenous keloid matrix was observed both in histology and by qPCR of whole keloid tissue mRNA. The overexpression of fibronectin (FN), collagen I (COL1A1) and collagen III (COL3A1) in keloid tissue was statistically significant and confirmed previous findings in the literature (Abergel *et al.*, 1985; Babu, Diegelmann and Oliver, 1989; Clark, 1990). Notably, keloid expression of these factors was variable, and the two patients that showed the highest expression of fibronectin also showed the highest expression of the two collagen types. The overexpression of collagens as well as the unusual arrangement of collagen fibres in keloid tissue seen in histological staining could support the importance of biomechanics in keloid pathology. A hypothesis published in 2016 postulated that the 'stiffness gap' caused by excessive and abnormal ECM deposition in keloids may drive cellular behaviour such as migration and proliferation that can cause keloid growth (Huang *et al.*, 2016). Certainly mechanosensing by cells in the skin is integral for successful wound healing and the abnormal ECM environment found in keloid scars could affect cell behaviour and contribute to the activation and differentiation of fibroblasts to a myofibroblast phenotype (Hinz, 2009; Huang *et al.*, 2012; Kenny and Connelly, 2014).

The lower level of ACTA2 (α-SMA) mRNA seen in keloid tissue compared to normal skin is not surprising, given that firstly there are such varied reports of α-SMA positivity in keloid scars and secondly that normal skin is highly vascularised and these vessels stain as α-SMA positive (personal communications/unpublished data, Zoe Drymoussi).

With an improved understanding of the fibrotic environment of keloid tissue, BET proteins were then analysed both at the mRNA and protein level in keloid tissue compared to normal controls. qPCR analysis of normal skin and keloid whole tissue mRNA did not indicate any difference in expression of BRD3 between normal and keloid scar and equally analysis of BRD3 protein in normal skin and keloid dermal tissue lysates by western blot showed similar levels of expression with a single keloid sample showing higher expression. Interestingly, the

keloid that showed the strongest BRD3 expression also showed the greatest expression of BRD2 which may not be surprising as these two BET family members are thought to have overlapping functions (Leroy, Rickards and Flint, 2008). BRD3 has the least amount of literature available (**Table 3.4**) and therefore much less is known about its function in both health and disease. Some level of expression was detected in all samples suggesting it has some importance in normal skin biology.

***Table 3.4* - Number (#) of publications with BRD2, BRD3 or BRD4 in the title.**

| Keyword | # of publications with keyword in the title |
|---|---|
| BRD2 (or RING3) | **66** |
| BRD3 | **11** |
| BRD4 | **265** |

*Data based on PubMed search (11/06/17) for 'Brd2[title] OR RING3[title]', 'Brd3[title]' or 'Brd4[title]'.*

Slightly higher levels of BRD4 mRNA expression was observed in normal skin compared to keloid scar and this also held true at the protein level where 4 out of 6 normal skin dermal lysates had higher BRD4 protein by western blot. BRD4 expression patterns were then examined by IHC of normal skin, normal scar and keloid tissue, which revealed positivity in almost all cells in the dermis of all three types of tissue, although staining appeared most intense in normal skin. As cells with fibroblast morphology were observed to be BRD4 positive, BRD4 mRNA was also analysed in normal skin and keloid fibroblasts, both of which were positive although not different which may be attributable to the culturing method, which will be discussed later.

The high levels of BRD4 in normal skin tissue again suggests that BRD4 is likely to have a physiological role in the skin. Indeed sustained *Brd4* silencing in mice causes a substantial skin defect through an unknown mechanism, with epidermal hyperplasia, increased sebaceous gland size and increased numbers of (and abnormal formation of) hair follicles (Bolden *et al.,* 2014). A possible mechanism for the hair phenotype is that Brd4 is thought to be important for stem cell differentiation (Micco *et al.*, 2014). Our IHC showed lots of BRD4 positivity surrounding the bulge region of the hair follicle where such stem cells would reside (data not shown), and the lack of appendages including hair follicles in keloid scar may explain the lower amount of Brd4 compared to normal skin.

The most striking difference in BET expression was seen for BRD2. qPCR analysis of whole tissue mRNA indicated substantial overexpression of BRD2 (up to 100 fold higher than normal

skin) in 3 out of 5 keloid samples, although this was not statistically significant. Normal skin mRNA showed less variation, even the normal skin sample from a keloid patient, isolated adjacent to keloid scar, had expression levels in line with other normal skin samples. Similarly, when normal skin and keloid scar tissue were lysed and analysed for BRD2 protein by western blot, there was higher BRD2 expression in a number of keloid samples. Notably, in instances where two keloids from the same patient were assessed for expression, both keloids had some level of overexpression which could suggest there is a common mechanism in the formation of multiple keloids. There was no obvious clinical explanation for keloid dermis that showed strong BRD2 expression, such as anatomical site or level of inflammation (assessed by examining H&E histology) as BRD2 was expressed by immune cell clusters in our IHC and it is important in immune cell function (Belkina, Nikolajczyk and Denis, 2013; Belkina *et al.*, 2014). The relatively low BRD2 protein expression in normal skin by western blot was not unexpected given that BRD2 has been linked to progression of the cell cycle (Sinha, Faller and Denis, 2005) and in normal skin there is very little turnover of fibroblasts in the dermis (McGrath, Eady and Pope, 2004).

Analysis of BET proteins was somewhat complicated by the appearance of multiple bands of unexpected sizes, in particular a 60kDa BRD2 band that was strongly expressed in a subset of keloid samples. This was further explored in a western blot with a range of cell lysates which indicated further differential expression of BRD2 bands with sizes ranging between less than 30kDa and more than 250kDa. Consistent with this, a number of commercial antibodies report band sizes that are not predicted but are thought to be the consequence of post-translational modification. For example, a BRD2 antibody retailed by Sigma (cat no. 07-1526) reports an observed band at ~110kDa with an uncharacterised band in some lysates at ~90kDa described to be due to phosphorylation at different sites on the protein. This demonstrates the difficulty presented by using BET antibodies and interpreting resulting banding patterns. Attempts to immunoprecipitate with the rabbit polyclonal BRD2 antibody, to identify the multiple BRD2 bands with mass spectrometry (as well as other potential interacting proteins) were unsuccessful (Appendix, **Figure 7.2**).

The rabbit polyclonal BRD2 antibody has been validated as detecting recombinant full length BRD2 (GSK) but is raised against an epitope between the two bromodomain regions of the protein and so it is possible that the bands represent different isoforms and/or post translational modifications of the proteins. Indeed, when focussing on BRD2 in particular, there are at least 8 possible protein coding isoforms (**Figure 3.12**). Protein sequences from each isoform reported on Vega/Havana were entered into a publicly available online tool for protein domain prediction (ScanProSite). BRD2-023 could account for the 60kDa band observed in the tissue lysates, which notably lacks the nuclear localisation signal domain (NLS) and the N Extra Terminal domain (NET) which is thought to be important for DNA binding

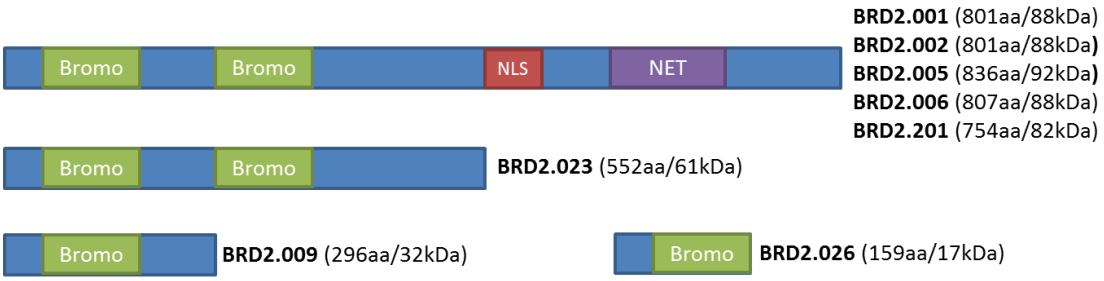



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***Figure 3.12* - Schematic of possible protein-coding BRD2 isoforms.** Protein sequence information for each BRD2 isoform reported on Ensembl.org was entered into the ExPASy tool ScanProSite to generate a structure of predicted motifs.

This could explain some cytoplasmic presence of BRD2 which was observed in IF staining of fibroblasts, which was also seen in staining of rheumatoid arthritis (RA) fibroblast-like synoviocytes (Xiao *et al.*, 2015). The consequence of BRD2 in the cytoplasm is currently unknown; however, given that BETs can interact with non-histone proteins through acetylated lysine, there may be relevant protein interactions occurring in the cytoplasm. Also, similarly to the BRD4 short isoform (Alsarraj *et al.*, 2013), this truncated version of BRD2 may have a more promiscuous binding pattern to histones, which in turn may drive different patterns of gene expression.

Efforts to investigate the expression profiles of different isoforms were unsuccessful within the span of this project, first through RACE-PCR and then through extensive qPCR attempts using primer sets to amplify various combinations of isoforms (in Appendix, below).

Two internal reverse primers were designed within the second exon of BRD2 whilst a RACE forward primer specific for the sequence that was ligated onto the 5' end of transcripts during cDNA preparation was used. This method theoretically should have allowed the amplification of multiple BRD2 isoform transcripts (including full length BRD2 and the 60kDa isoform of particular interest) which could be separated by electrophoresis and identified by size and/or sequencing.

qPCR primers were also designed with the help of Catriona Sharp (GSK) to amplify the different transcripts. Unique primers sets could be designed for BRD2-001 and BRD2-002 only (**Figure 3.12**), whilst other primer sets were designed to amplify different combinations of transcripts (detailed in Appendix, below). Due to technical challenges in controlling for primer efficiency across the different transcripts, it was not possible to make the detailed analysis of BRD2 isoforms that was planned. If differential isoforms, such as the 60kDa BRD2 isoform, are indeed present in disease, then cloning and overexpression of specific constructs in dermal

fibroblasts could help to understand their functions and in turn importance in disease, as well as gaining a better understanding of the function of BET protein domains.

A commercial BRD2 IHC antibody from Bethyl Lab was used for the IHC analysis as pilot experiments showed the GSK BRD2 antibody did not work (data not shown). This antibody was raised against an epitope at the C terminal end and therefore should only detect the full length protein. The BRD2 positive cells within the dermis were interpreted to be fibroblasts based on their location and morphology, although this could not be confirmed by co-staining due to the lack of specific human fibroblast marker. Interestingly, there was variable expression of BRD2 in dermal fibroblasts in some normal skin samples, indicating the possibility of fibroblast subsets that are BRD2 negative and positive. Our understanding of fibroblast heterogeneity is expanding with the discovery of a number of progenitor fibroblast populations in mice that are likely to have different contributions to wound repair in particular (Driskell and Watt, 2014; Rinkevich *et al.*, 2014). However, similar work is still ongoing in human skin (Phillippeos & Watt, personal communication). The comparatively more homogenous expression of BRD2 seen in the keloid dermal fibroblasts by IHC may reflect an expansion of the BRD2 positive fibroblasts. In future work, it would be helpful to work up a Ventana staining method with a different coloured substrate to allow co-staining (e.g. for CD45) to better understand the cell populations that are contributing to dermal expression.

As it appeared to be mainly dermal fibroblasts that were responsible for BRD2 expression in the dermis, western blotting was carried out on cultured NDFs and KDFs isolated from human samples. This showed no difference in the expression of BRD2 protein and indeed no difference in the mRNA level of BRD2, BRD3 or BRD4, including the ratio between the short and long BRD4 isoforms. The lack of difference seen could be due to the artificial environment of cell culturing whereby conditions are formulated to promote growth and expansion of cells rather than retaining *in vivo* characteristics. We believe this environment, on a tense plastic substrate in the presence of serum may cause NDFs to become less 'normal' and more scar like. Foetal calf serum is often added as a supplement of culture media and is a key driver of cell proliferation (Ryan, 1979) however some reports describe that cells in contact with serum are demonstrating a damage-like response, as *in vivo* serum is involved in the clotting response after wounding and contains many growth factors and hormones that may induce proliferative behaviour (Chang *et al.*, 2004).

To better understand what factors may regulate BRD2 expression *in vivo*, which may be missing in our culturing conditions, cells were treated for 72 hours with a panel of keloid associated factors (IL-6, IL-17A, TGF-β1 and PDGF-BB) or inflammatory type factors (LPS, PMA, IFNγ and TNFα). All of these keloid associated factors caused a small increase in BRD2 protein in

the NDF line, however the inflammatory factors LPS and TNFα induced higher levels of BRD2, which included another band at ~60kDa.

LPS stimulation in some way models a bacterial infection of cells by stimulation through TLR4, which has been shown to activate inflammatory gene expression in hypertrophic scar dermal fibroblasts through NF-κB activation (Wang *et al*. 2010). *In vivo*, TNFα may be produced by a range of immune cells, activated macrophages in particular (Olszewski *et al.*, 2007), which have been reported to be present in keloids (Bagabir *et al.*, 2012a). Keratinocytes can also produce TNFα (Bashir, Sharma and Werth, 2008), and this may be aberrant in keloids where the cells are abnormally differentiated (Limandjaja *et al.*, 2017). It would be interesting to repeat the LPS and TNFα stimulation in KDFs to see whether similar induction of BRD2 expression occurs or whether the expression may be regulated differently in these cells. It is unclear why conditioned media from keloid *ex vivo* cultures is able to reduce BRD2 protein levels in a KDF line after 24 hours of culture; it may be due to a range of factors in the supernatant that we have not tested that may be able to negatively regulate expression. Better characterisation of the secretome of keloid tissue in the future may aid with further studies of BRD2 regulation.

It would be informative to examine the direct transcriptional consequences of Brd2 in keloid vs normal skin. One strategy to do this would be to analyse chromatin binding patterns of BRD2 by ChIP-Seq, including association with super-enhancer regions. Inflammation has been shown to cause BRD4 recruitment to chromatin in the form of super-enhancers, which could drive pathological gene expression as has previously been reported in other cell types (Brown *et al.*, 2014; Xu and Vakoc, 2014). Super enhancers have been shown to regulate a range of processes from cell fate to disease progression (Hnisz *et al.*, 2014; Micco *et al.*, 2014) although limited information can be gleaned from existing datasets as BET protein activity is thought to be very cell and stimuli specific (personal communications with GSK). It would also be interesting to undertake proteomics on BRD2 immunoprecipitation on whole keloid tissue or cells to identify non-histone targets which may be different in health vs disease. The following chapters explore BET protein activity in keloid fibroblasts by examining the cellular consequences of BET protein inhibition, which may also shed light on whether these proteins could be targets of therapeutic value in a keloid context.

# Chapter 4: Evaluating the effect of BET protein inhibition on disease associated cell behaviours

## 4.1. Introduction

Keloid derived cells, in particular fibroblasts (KDFs) appear to have altered characteristics and behaviour that contribute to the disease; KDFs have been shown to be more proliferative (Satish *et al.*, 2004; Blažić and Brajac, 2006; Ghazizadeh *et al.*, 2007), more resistant to apoptotsis (Chipev *et al.*, 2000; Ishihara *et al.*, 2000), more inflammatory (Messadi *et al.*, 2004; Fujiwara, Muragaki and Ooshima, 2005; Uitto, 2007), more contractile (Chipev *et al.*, 2000) and more matrix producing (Babu, Diegelmann and Oliver, 1989; Syed *et al.*, 2011).Targeting epigenetics may offer a novel strategy to influence multiple cell behaviours and "normalise" the phenotype of disease cells to a healthy state.

There is strong evidence for epigenetic changes in disease particularly in inflammatory conditions (Tough *et al.*, 2016) with increasing interest in targeting this therapeutically. Keloid scars specifically have been reported to be epigenetically changed compared to normal controls, with one particular study focussing on keloid fibroblasts which showed differential DNA methylation and histone acetylation compared to normal controls (Russell *et al.*, 2010). Although there has been no analysis of the wide-scale acetylome in keloid fibroblasts the Russell *et al* study did show a decrease in specific keloid-gene transcription, such as IGFBP5 and JAG1, after treatment with the histone deacetylase (HDAC) inhibitor TSA. Interestingly overexpression of p300, an enzyme with the opposite activity (histone acetyl transferase, HAT) also had an anti-fibrotic effect in scleroderma patient fibroblasts cultured with TGF-β1, suggesting that differential acetylation at specific genomic loci, be it increased or decreased, can modulate the expression of fibrosis relevant target genes, such as collagen genes. Given the association of BET proteins with acetylated lysine residues on proteins including histones, this may indicate that BET proteins could have differential activity in diseases including keloid scarring.

Indeed, small molecule BET inhibitors, which bind to and block BET bromodomain activity, have shown efficacy in a wide range of disease models, many of which have relevant features to keloid scarring. These studies have also importantly indicated that the efficacy of BET inhibition does not exclusively dependent on the overexpression of BET proteins in disease. Instead BET proteins may influence disease through different patterns of gene expression which can be altered or even reverted to a 'normal' phenotype by BET protein inhibition.

BET inhibition has shown positive effects in a range of cancer models, namely through the inhibition of proliferation and induction of apoptosis (Dawson *et al.*, 2011; Chaidos *et al.*, 2014; Gallagher *et al.*, 2014). This could offer an advantage in the treatment of keloid scars where cells are thought to be persistently proliferative and/or resistant to apoptosis, which is a process that is essential to normal wound healing resolution (Sayah *et al.*, 1999; Ishihara *et al.*, 2000).

In addition to this, BET inhibition has been shown to be anti-inflammatory, in part by causing a decrease in proinflammatory cytokines, and it is via this mechanism that the drug is thought to improve outcomes in models of retinal degenerative disease (Li *et al.*, 2017), sepsis (Belkina, Nikolajczyk and Denis, 2013) and rheumatoid arthritis (RA) (Klein *et al.*, 2014). More recently, there has been increasing evidence that BET inhibition showing beneficial effects in several organ fibrosis models including in the kidney (Zhou *et al.*, 2017), liver (Ding *et al.*, 2015) and lung (Tang *et al*. 2013a), by downregulating fibrosis related genes, including those related to the deposition of matrix such as collagen and fibronectin.

This section of work utilised one such inhibitor called I-BET151 (GlaxoSmithKline) as a pre-clinical tool to interrogate the effect of BET protein inhibition in both NDFs and KDFs on both protein/gene read-outs and corresponding phenotypic behaviours.

I-BET151, also named GSK1210151A, was created by GlaxoSmithKline to target BET family proteins, and was first published in a paper showing efficacy in models of an aggressive and difficult to treated form of leukaemia called mixed lineage leukaemia (MLL) (Dawson *et al.*, 2011). The inhibitor caused cell cycle arrest and apoptosis in human and mouse MLL cell lines and provided a significant survival benefit in two different mouse models of MLL.

Given the plethora of effects of BET inhibitors reported on different cell types, the main objective of this chapter was to explore the effects of BET inhibition in a dermal fibroblast context. We used I-BET151 as a pre-clinical tool, treating NDF and KDF monolayers and evaluating the effect on four key cell behaviours:

1. Proliferation
2. Collagen gene expression
3. Contraction
4. Protease activity

In addition, a whole tissue biopsy *ex vivo* model (Bagabir *et al*. 2012b) was utilised to evaluate the effect of I-BET151 on a more complex cellular system, with particular focus on proteases.

## 4.2. Results

### 4.2.1. Proliferation is decreased in both normal and keloid dermal fibroblasts after pharmacological BET inhibition

Cell counting experiments were used to assess the effect of bromodomain inhibition by I-BET151 on the proliferation of primary dermal fibroblasts. Due to the relatively slow doubling time of dermal fibroblasts, counts were performed at 72 hours. In both cell lines a statistically significant decrease in cell number was seen after treatment with 1μM I-BET151 with around 50% less cells than the vehicle control on average (**Figure 4.1A**). To explore variations in sensitivity to BET inhibition between NDFs and KDFs, a titration of I-BET151 concentration was used for a number of biological replicates and this showed a concentration-dependent decrease in cell number of both NDFs and KDFs, with an effect even seen at a low dose of 1nM with around 75% of the cell number of the vehicle control (**Figure 4.1B**). Additionally I-BET151 treatment seemed to have a greater effect on KDFs, although this was not statistically significant (**Figure 4.1B**).

Curiously, a higher throughput method of measuring cell number by WST-1 assay was trialled but unsuccessful. WST stands for water soluble tetrazolium salt and is a colormetric assay which relies on the conversion of a dye from a colourless to a coloured substrate through cleavage by mitochondrial enzymes (Peskin and Winterbourn, 2000). This assay should in theory reflect cell number, however despite visual and cell count confirmation that there were less cells present after I-BET151 treatment, the WST-1 results remained equal across vehicle and drug treated conditions (data not shown).

No visible signs of cell death, such as floating cells or blebbing nuclei, were observed in response to I-BET151, but to confirm that this decrease in cell number indeed due to a decrease in proliferation, rather than cell death, cells were analysed for Ki67 staining which is a proliferation marker expressed in late G1, S, G2 and M phase of the cell cycle (Bologna-Molina *et al.*, 2013). Ki67 immunofluorescent staining of fibroblasts treated for 24 hours with 1μM I-BET151 or a vehicle control (0.01% DMSO) was performed and images were analysed in ImageJ to determine the percentage of Ki67 positive cells relative to the total number of cells as marked by DAPI (**Figure 4.2A**). A decrease in the percentage of Ki67/DAPI double positive cells after 1μM I-BET151 treatment was observed with further reduction at 10μM, with only ~35% of NDFs and ~20% of KDFs double positive at this dose, which showed statistical significance compared to the vehicle control (**Figure 4.2B**) although this was deemed to be

approaching a concentration that could give rise to off-target and potentially toxic effects (personal communications with GSK).

A wash-off experiment was also carried out to investigate the persistence of the anti-proliferative effect induced by I-BET151, (**Figure 4.3**). Both NDFs and KDFs showed a decrease in Ki67 expression after 24 hours of 1μM I-BET151 treatment, denoted as '0h'. Following removal of drug and thorough washing with PBS, the reduction in Ki67 persisted for less than 6 hours, indicating this is a reversible effect. Although this experiment was conducted on limited biological replicates, it seems that the decrease in KDF proliferation may persist longer after wash off compared to NDFs. Intriguingly in early KDF time points there appears to be an increase of GAPDH with I-BET151 treatment (**Figure 4.3B**) showing further evidence for a drug induced increase in metabolism as initially indicated by WST-1 assay (data not shown).

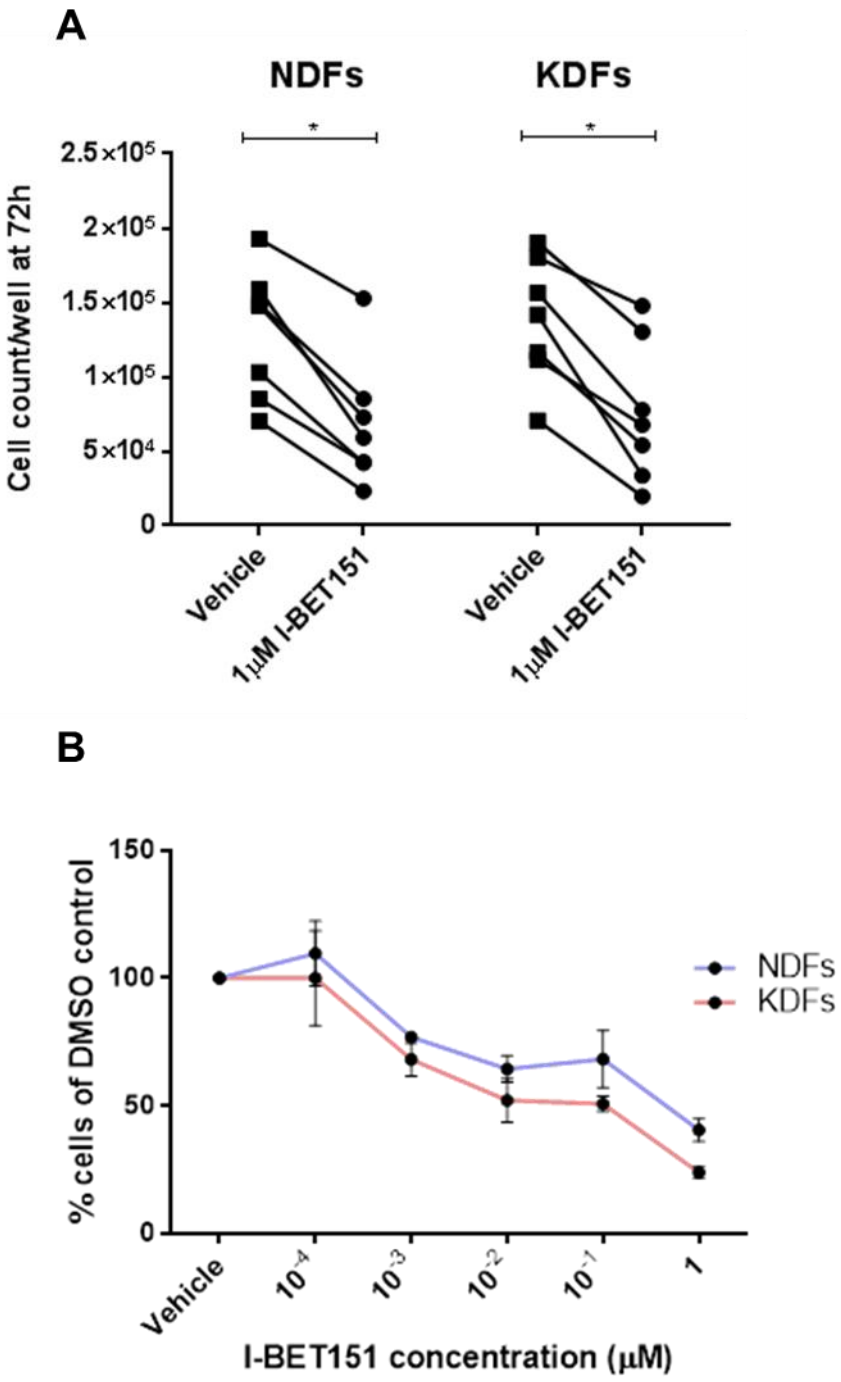



***Figure 4.1*** **- Dermal fibroblast proliferation is reduced after I-BET151 treatment.** (**A**)**:** Cell counts were taken at 72 hours after treatment with 0.01% DMSO vehicle or 1μM I-BET151 treatment, where dots linked with lines are paired samples, n=5, * = $p<0.05$ by Wilcoxon t-test. (**B**) Concentration dependent decrease in NDFs and KDF cell number after 72 hours of I-BET151 treatment compared to vehicle control (0.01% DMSO), n=3, error bars represent SEM

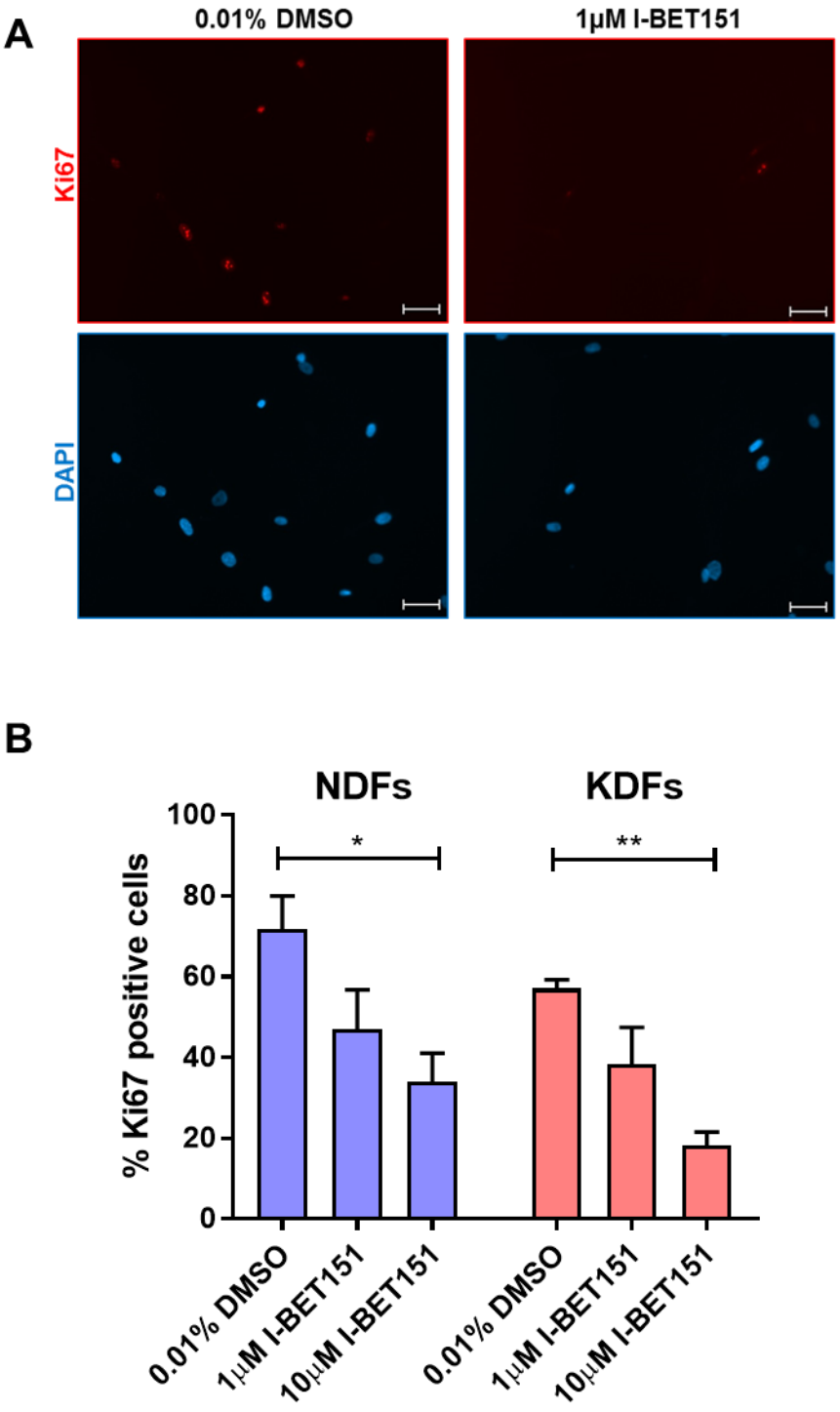



***Figure 4.2*** **- Decreased Ki67 positivity with I-BET151 treatment.** (**A**) Representative images of Ki67 (red) and DAPI (blue) immunofluorescent staining on NDF and KDF cell line after 24 hours of 1µM I-BET151 treatment. Scale bar = 20µm. (**B**) Quantification of Ki67 staining (% of Ki67+ nuclei / total nuclei (DAPI) after 24 hours of 1µM I-BET151 treatment in both NDF (n=4) and KDFs (n=3), error bars represent SEM. * = $p<0.05$, ** = $p<0.005$ by 2-way ANOVA with multiple comparisons.

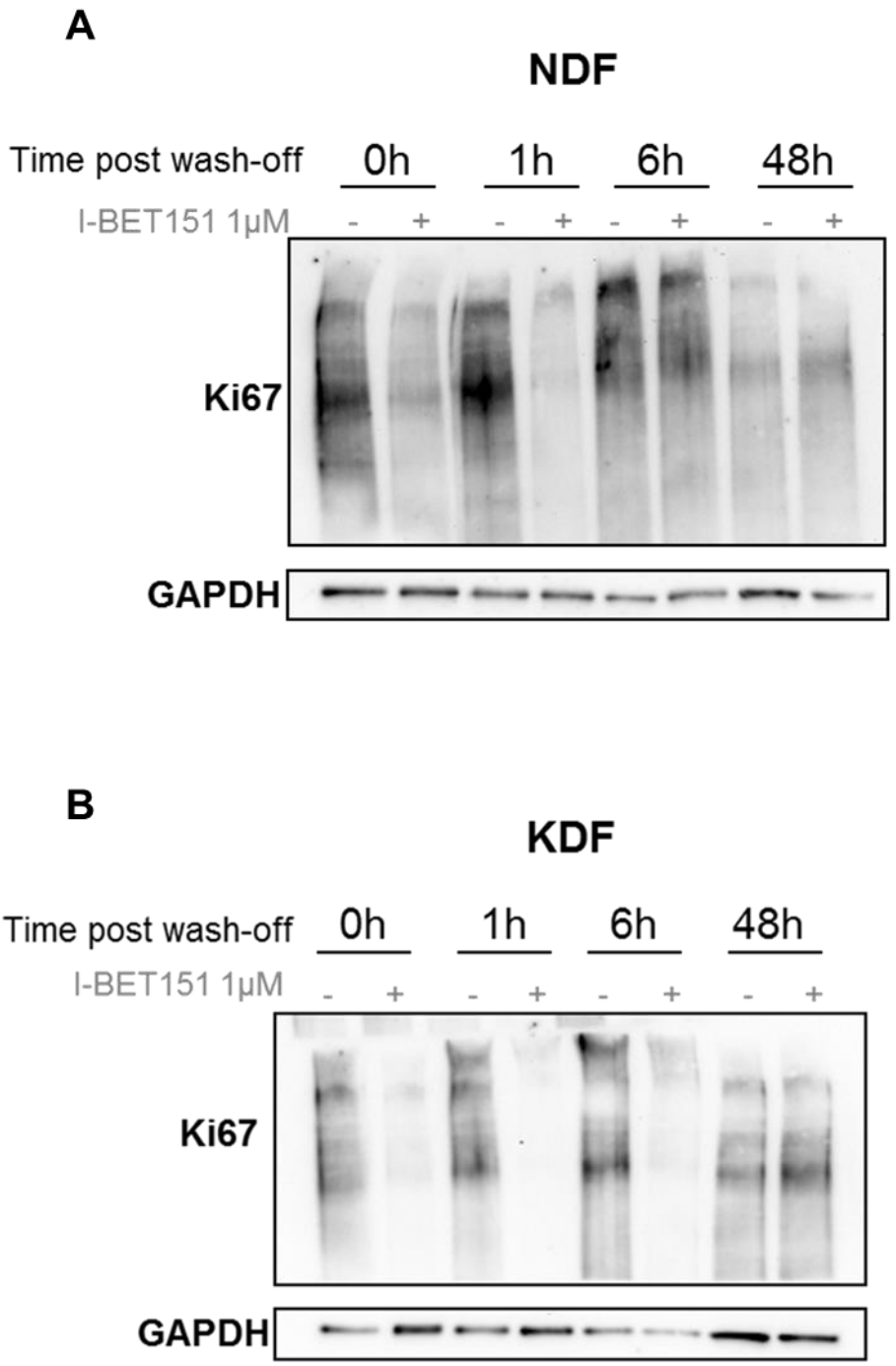



***Figure 4.3*** **- Recovery of dermal fibroblast proliferation after I-BET151 wash-off.** Representative western blots showing Ki67 expression after 24 hours 1μM I-BET151 treatment (0h) and the time course of Ki67 recovery after inhibitor wash-off in NDF (**A**) and KDF (**B**).

### 4.2.2. BET inhibition does not affect collagen expression

The production of collagen was also analysed, following previous observations that keloids are characterised by excessive collagen (**Figure 3.3**). Firstly, mRNA levels of two stereotypical scar associated fibrillar collagens were analysed using primers for COL1A1 and COL3A1 in NDF and KDF cultures treated for 48 hours with 1μM I-BET151 or vehicle control. This analysis showed that cells had a very heterogeneous response to drug treatment. 60% of NDFs and KDFs showed a decrease in COL1A1 (3 out of 5 biological replicates after I-BET151 treatment (**Figure 4.4A**). However, KDFs had a slightly more consistent decrease in COL3A1 after I-BET151 treatment with decreased expression in 5 out of 6 biological replicates, compared to 4 out of 6 NDFs (**Figure 4.4B**).

When secreted collagen I protein was measured by western blot in the supernatants of NDFs and KDFs (n=3) treated for 48 hours with I-BET151 or vehicle control, no difference was seen (**Figure 4.4C**). Contrary to what was expected,, KDFs actually showed less protein compared to NDFs.

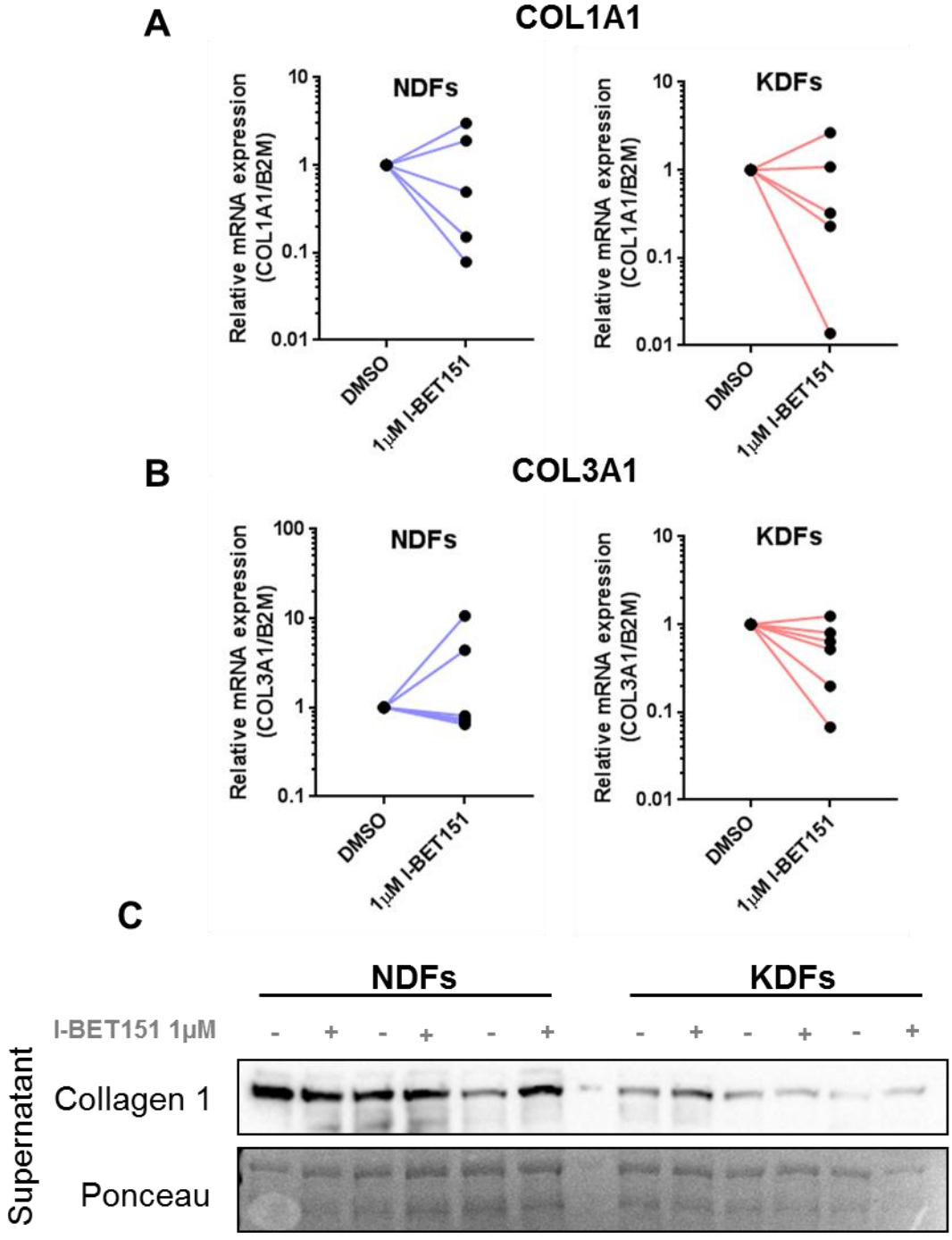



***Figure 4.4* - Effect of I-BET151 on collagen expression.** Collagen was analysed after 48 hours of treatment with 1μM I-BET151 or vehicle control. qPCR was used to analyse mRNA levels of two scar associated collagen transcripts, COL1A1 (**A**) and COL3A1 (**B**) using the standard curve reference and normalising to the reference gene B2M. Secreted collagen 1 was then measured in supernatants of cultures by western blot with Ponceau staining of total protein as a loading control, n=3.

### 4.2.2. Contraction is decreased in both normal and keloid dermal fibroblasts after BET inhibition

Contractility is another important scar associated cellular parameter that was measured in response to BET inhibition. Although contractility isn't explicitly reported to be increased in keloid dermal fibroblasts, there have been reports in other fibroses such as scleroderma that contractility is enhanced (Chen *et al.*, 2005). To assess dermal fibroblast contractility, a collagen gel contraction assay was used with low passage fibroblasts (less than 3 passages) in an effort to retain the *in vivo* phenotype of the cells as pilot studies showed these cells tended to be more contractile. Fibroblast-seeded collagen gels (n=7 biological replicates) were free-floated in media containing either DMSO (vehicle) or 1µM I-BET151. At 72 hours, a significant reduction in gel size was seen in vehicle treated replicates as opposed to I-BET151 treated replicates that retained much of their original size (**Figure 4.5**). A subset of KDF lines showed increased contractility compared to NDFs but this was not consistent across all patient samples tested.

Cell contractility in scar associated fibroblasts can be mediated by α-SMA, which is often referred to as a myofibroblast marker (Hinz *et al.*, 2007). Therefore fibroblast monolayer cultures treated with 1µM I-BET151 or DMSO vehicle control for 48 hours were harvested for RNA extraction. After reverse transcription, levels of ACTA2 (α-SMA transcript) and the reference gene B2M were measured by qPCR. This analysis showed a dramatic decrease in ACTA2 mRNA in both NDFs and KDFs after 48 hours of treatment with 1µM I-BET151 (**Figure 4.5**).

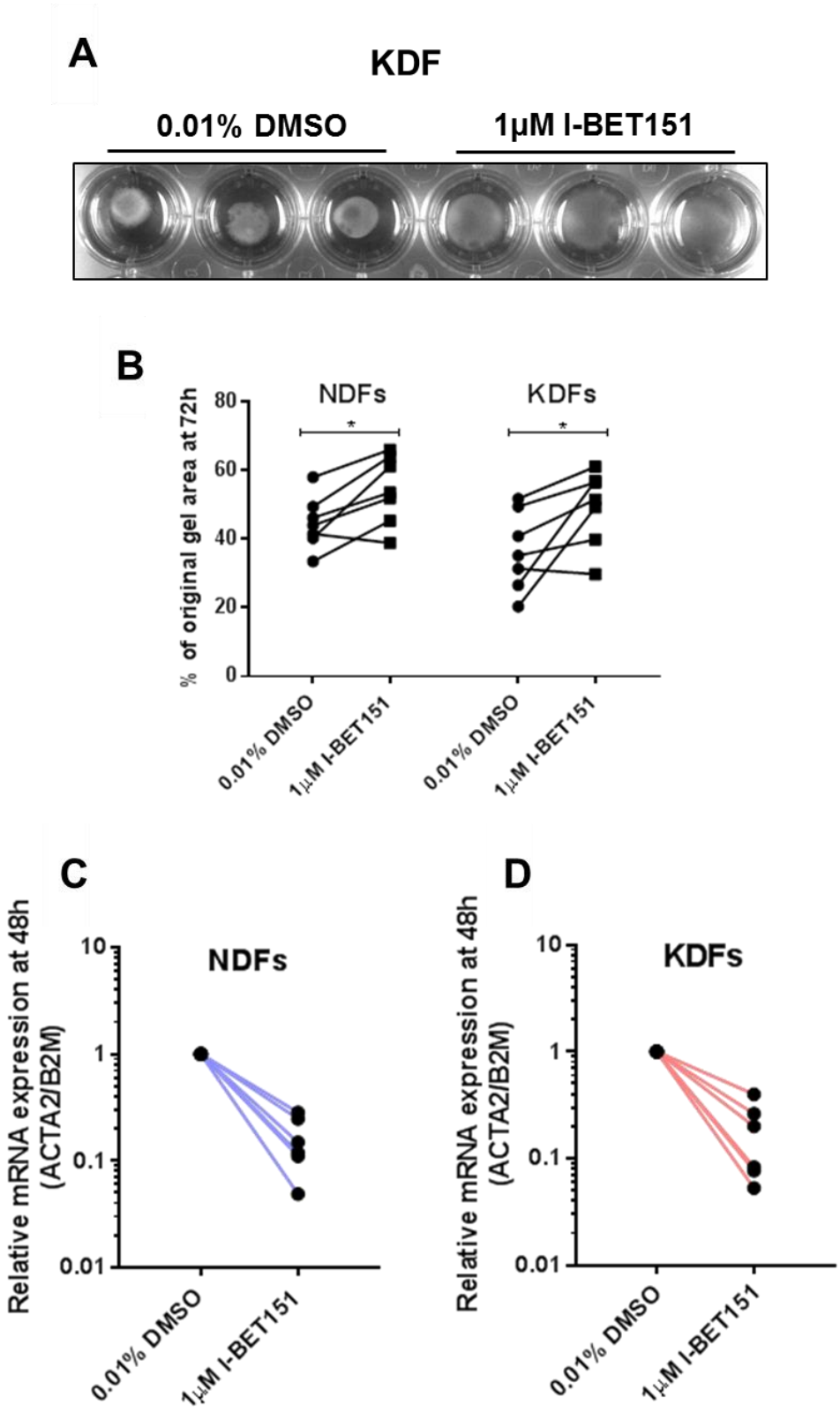



***Figure 4.5* - I-BET151 decreases collagen gel contraction with an associated reduction in α-SMA expression.** (**A**) A representative gel contraction assay +/- I-BET151 with a keloid fibroblast cell line pictured after 72 hours of contraction. (**B**) Summary of experiments carried out for n=7 NDFs and KDFs with gel size measured at 72 hours (* =$p<0.05$ by Wilcoxon's t-test). (**C**) qPCR for α-SMA in n=5 NDFs and KDFs after 48 hours of I-BET151 treatment, relative to the reference gene B2M, normalised to the untreated control where lines represent different patient isolations.

### 4.2.3. Decrease in protease expression after BET inhibition

In order to further probe the effects of BET inhibition on keloid fibroblasts and more widely on keloid tissue, an *ex vivo* culturing technique was used (Bagabir *et al*. 2012b). The model is a 4mm ø biopsy of tissue maintained in a media-collagen gel, with the epidermal surface at the air-liquid interface with a small volume of media on the surface of the gel which is replenished every 2-3 days. In this case, both the media-collagen gel and the media were supplemented with 1µM I-BET151 or DMSO as a vehicle control.

The original paper concluded that this culturing technique was capable of maintaining tissue health and integrity for up to 6 weeks (Bagabir *et al*. 2012b). The group then used this model to evaluate the effect of pharmacological compounds and siRNAs, showing effects such as tissue shrinkage, increased apoptosis, decreased proliferation, reduced inflammation and reduced collagen production (Syed *et al.*, 2013).

When the model was used in our lab, the first and most remarkable observation was a sporadic degradation of the collagen-media gel surrounding some tissue biopsies after 1-2 weeks of culture (**Figure 4.6**). Anecdotally, this observation occurred in both tissue types but with more prevalence in keloid samples. Notably, this effect was always prevented with 1µM I-BET151.

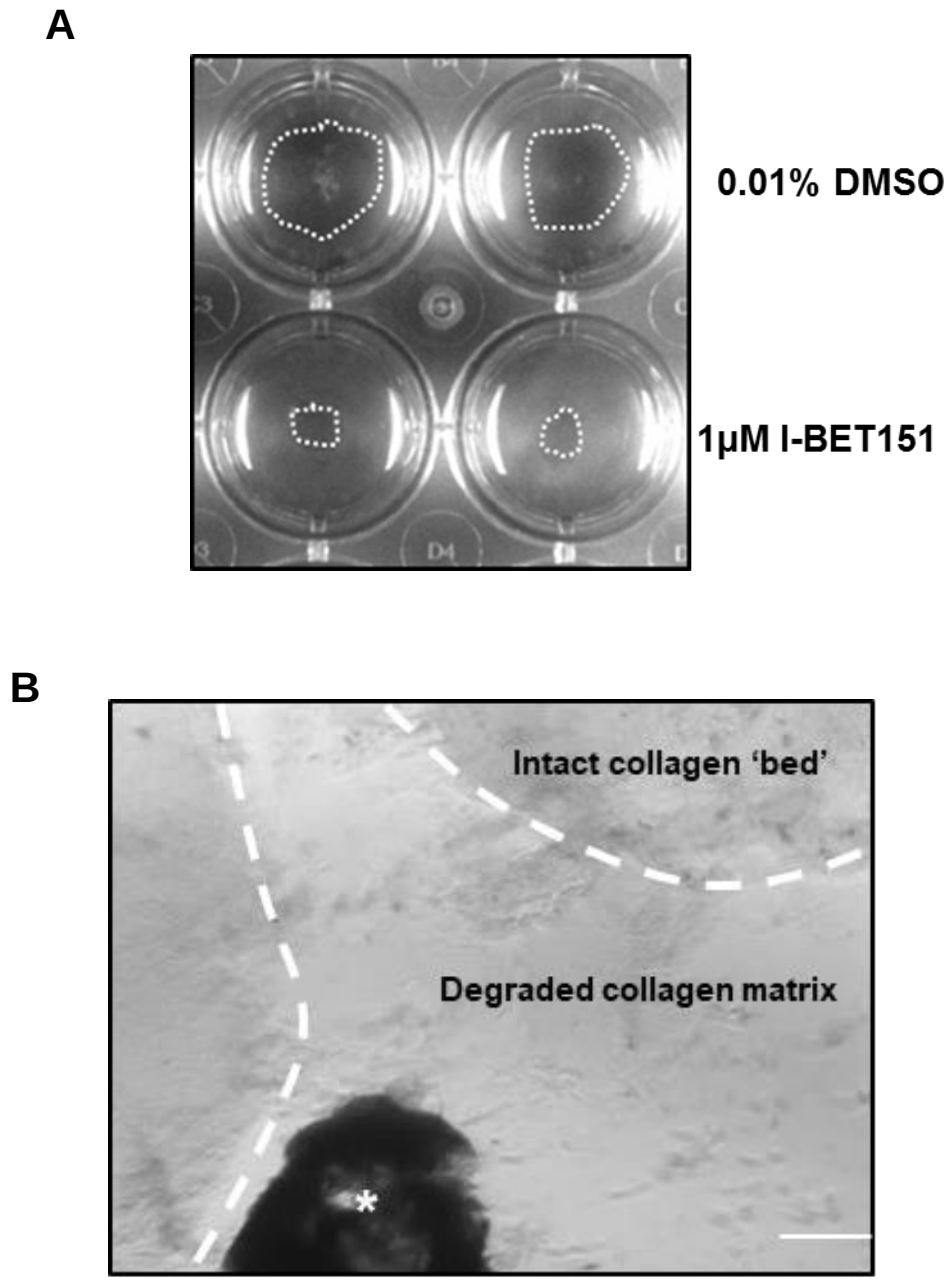



***Figure 4.6* - Illustrative examples of the degradative nature of keloid *ex vivo*s, which was prevented with I-BET151 treatment.** (**A**) Example image of degradation after the keloid tissue biopsy was removed from the collagen 'bed', conditions were performed in technical duplicate with vehicle control 0.01% DMSO (top row) or 1µM I-BET151 (bottom row). (**B**) Image captured on light microscope showing keloid tissue biopsy edge (*) and surrounding degradation of collagen gel (hatched line = edge), scale bar = 500µm

To investigate the mechanism behind this striking observation of tissue being released from the collagen bed, and I-BET151's ability to block this, supernatants were taken from these cultures after 24 hours of treatment with 1µM I-BET151 or vehicle control. 24 hour supernatants were also taken from fibroblast monolayer cultures with vehicle vs 1µM I-BET151 treatment.

These supernatants were used to probe the hypothesis that the degradation seen in the *ex vivo* cultures may be as a result of proteolytic activity, and that BET inhibition may be able to skew the protease balance to prevent this degradation. Therefore, MMPs and TIMPs were measured by a Luminex assay, a magnetic bead, antibody based multiplex assay with a similar protocol to an ELISA but allowing for the detection of a range of target analytes. Specifically, MMP1, 3, 7, 8, 9, 10, 12, and 13, and TIMPs 1, 2, 3, and 4 were measured. This technology detected pro and active forms of assayed MMPs, as well as TIMP complexed MMPs so was a measure of overall levels. When examining levels of MMPs and TIMPs, it was apparent that keloid tissue showed a trend for higher amounts of almost all MMPs, except MMP7, and similar amounts of TIMPs, compared to normal skin (**Figure 4.7A**). In contrast, monolayers of KDFs generally had lower MMP production than NDFs (**Figure 4.7B**).

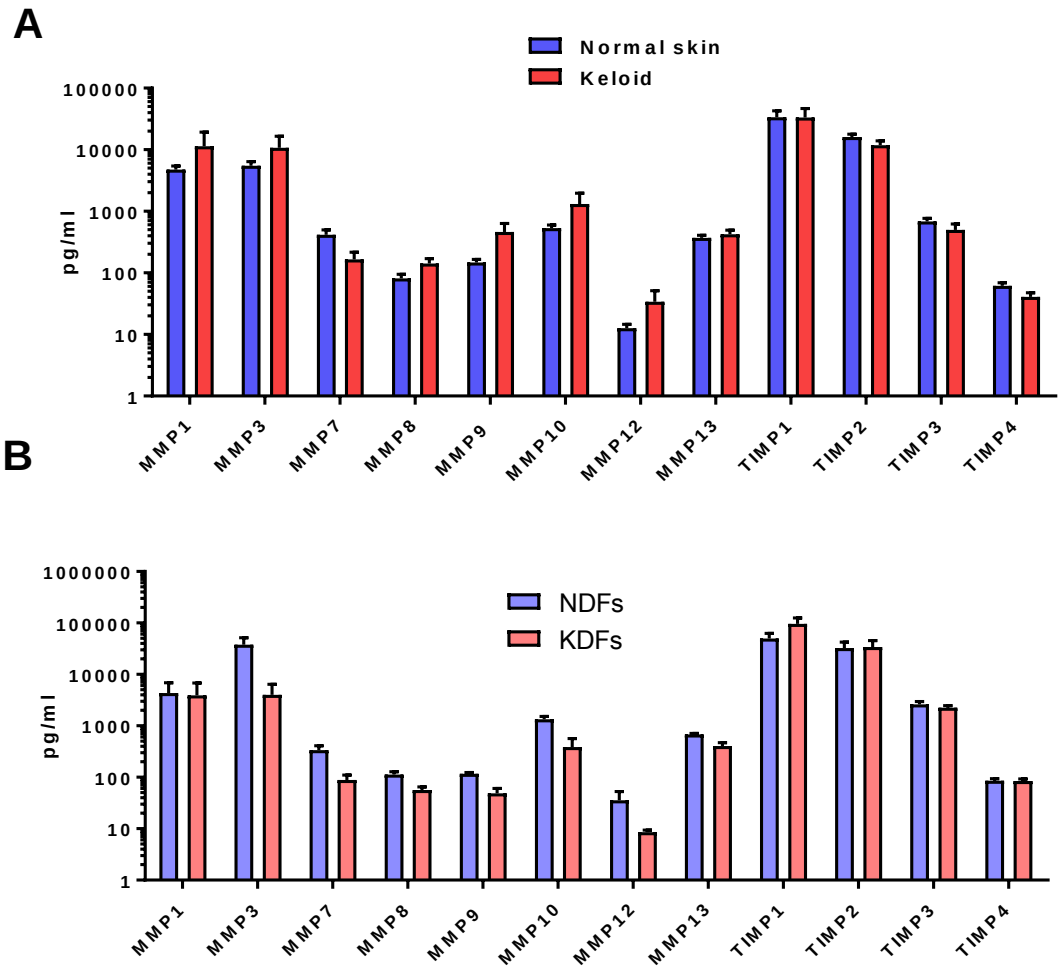



***Figure 4.7* - Baseline MMP and TIMP production in tissue *ex vivo* models.** Summary of Luminex data of all assayed analytes in 24 hour supernatant from 4mm tissue *ex vivo* model embedded in collagen with 200µl of media, n= 6 (**A**) and fibroblast cultures, seeded at 4 x $10^4$ cells per well in a 12 well plate with 500µl media, n = 3 (**B**). Bars represent mean +/- SEM analysed by Mann Whitney testing.

In addition to highlighting potentially greater proteolytic activity in keloid *ex vivo* cultures compared to normal skin *ex vivo* cultures, the results of the *ex vivo* experiment also suggested that this could be limited by I-BET151 treatment. Consistent with this, Luminex analysis on both *ex vivo* and fibroblast cultures treated with I-BET151 or vehicle control indicated a general decrease in a number of MMPs and an increase in a number of TIMPs after I-BET151 treatment (**Figure 4.8** and **Figure 4.9**). There were high levels of heterogeneity in both normal skin and keloid scar, in terms of starting level of analyte and in response to I-BET151. To visualised this information as fold change, the data was normalised by dividing each I-BET151 result by the paired vehicle control (**Figure 4.8B**).

MMP-9 showed the most consistent decrease after I-BET151 with almost all samples (10 out of 12, across both normal skin and keloid *ex vivo* cultures) (**Figure 4.8A)**. In fact the two keloid samples that produced a particularly high level of MMP-9 were the most drastically affected with around a 10 fold decrease. TIMP-1 showed the most consistent increase after I-BET151 with 11 out of 12 samples showing a higher concentration with treatment. However, upon thorough statistical analysis, there were only a few analytes that showed a significant change after I-BET151 treatment and these were not consistent between tissue types. Significant decrease by parametric testing was seen in MMP3, MMP9 and MMP12 in normal skin, whereas no significant changes were seen by parametric testing in keloid, although MMP8 and TIMP1 were significantly changed by non-parametric testing in keloid (**Table 4.1**).

When the same analysis was repeated for supernatants from the fibroblast cultures, there was less heterogeneity, and a clearer effect of I-BET151 treatment. Specifically, I-BET151 treatment decreased MMPs with a more dramatic response in KDFs (**Figure 4.9B**). Statistics are not displayed for fibroblast cultures due to the limited biological replicates.

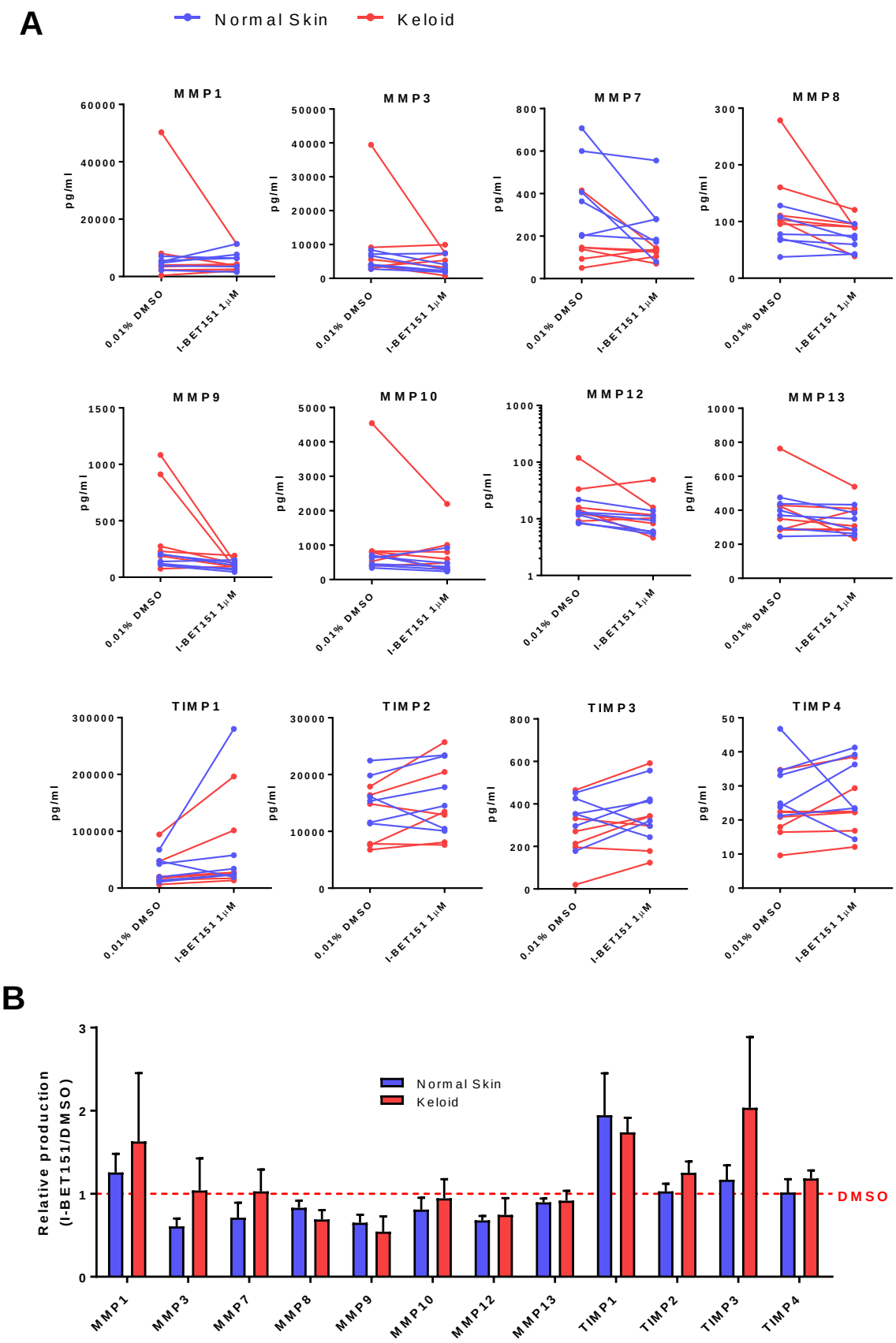



***Figure 4.8* - MMPs and TIMP production in normal skin and keloid tissue *ex vivo* cultures.** (**A**) Summary of Luminex data of all assayed analytes in normal skin and keloid tissue with 0.01% DMSO or 1µM I-BET151 treatment for 24 hours and (**B**) and change in expression after 24 hours of I-BET151 treatment (I-BET151 normalised to vehicle control), mean +/-SEM, n=5.

***Table 4.1* - P values for effect of I-BET151 on *ex vivo* cultures**.

| | Normal Skin | | Keloid | |
|---|---|---|---|---|
| | **Para** | **Non Para** | **Para** | **Non Para** |
| **MMP1** | 0.26 | 0.44 | 0.35 | 1.00 |
| **MMP3** | 0.03 | 0.06 | 0.40 | 0.69 |
| **MMP7** | 0.11 | 0.15 | 0.39 | 0.53 |
| **MMP8** | 0.06 | 0.09 | 0.12 | 0.03 |
| **MMP9** | 0.02 | 0.06 | 0.11 | 0.06 |
| **MMP10** | 0.33 | 0.31 | 0.35 | 0.44 |
| **MMP12** | 0.01 | 0.03 | 0.37 | 0.44 |
| **MMP13** | 0.08 | 0.09 | 0.30 | 0.31 |
| **TIMP1** | 0.31 | 0.31 | 0.12 | 0.03 |
| **TIMP2** | 0.75 | 0.69 | 0.12 | 0.22 |
| **TIMP3** | 0.54 | 0.69 | 0.09 | 0.16 |
| **TIMP4** | 0.85 | 1.00 | 0.12 | 0.06 |

Parametric and non-parametric (Wilcoxon's) t-tests were conducted on the Luminex data for vehicle vs I-BET151 controlled paired samples, n=5. Darkened cells are significant ($p<0.05$).

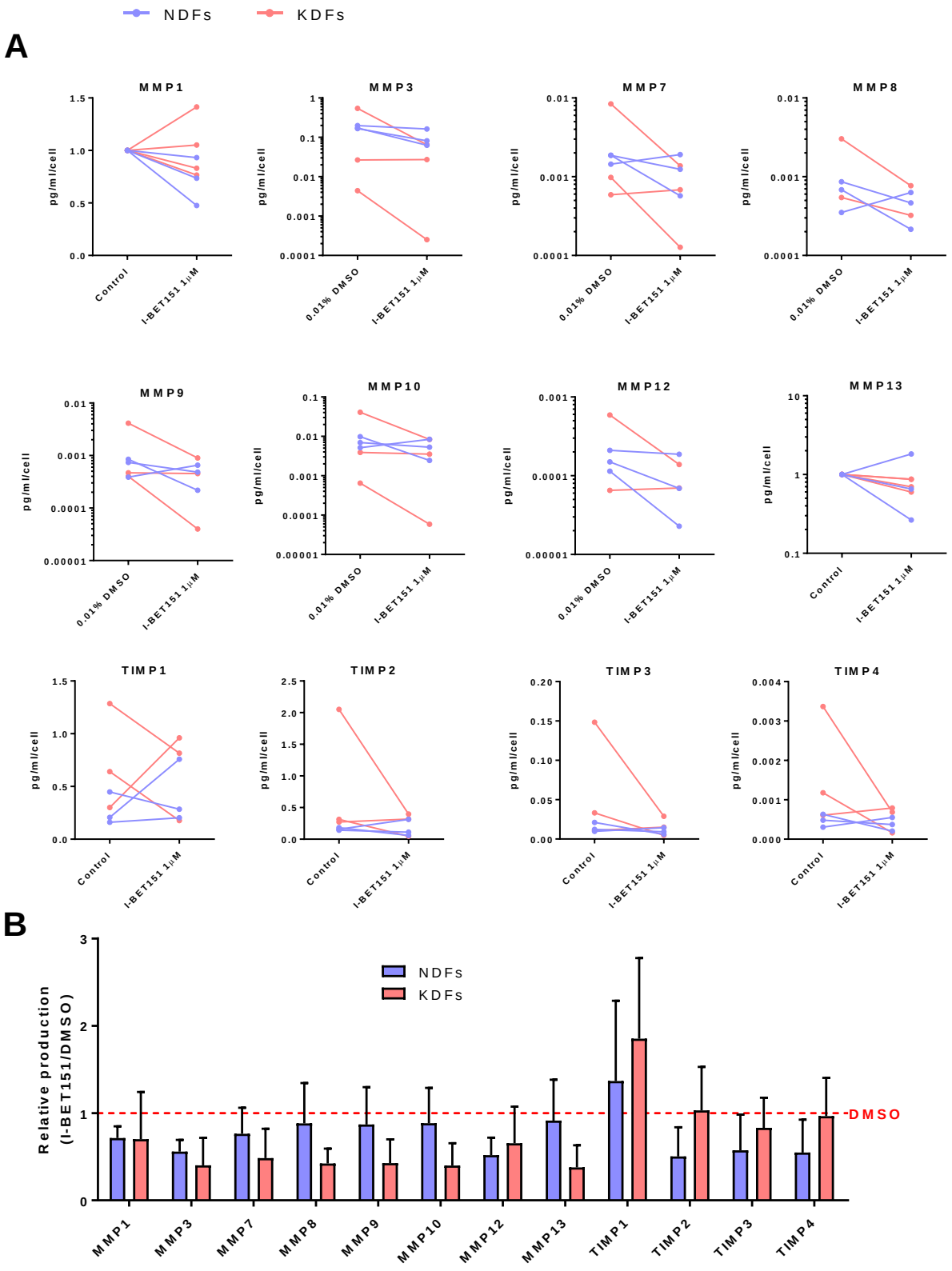
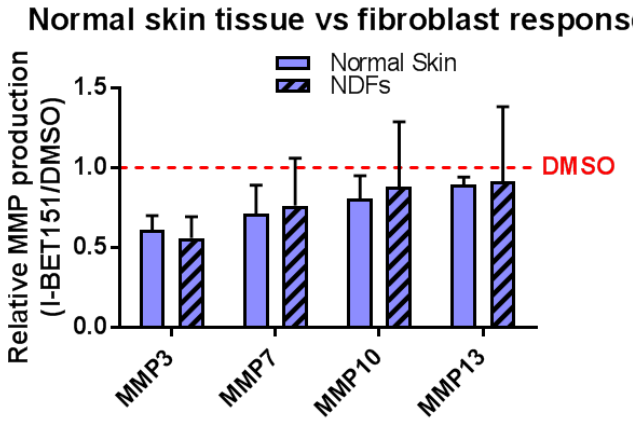



***Figure 4.9* - MMP and TIMP production in NDF and KDFs.** (**A**) Summary of Luminex data of all assayed analytes in NDFs and KDFs with 0.01% DMSO or 1µM I-BET151 treatment for 24 hours and (**B**) and change in expression after 24 hours of I-BET151 treatment (I-BET151 normalised to vehicle control), mean +/-SEM, n=3.

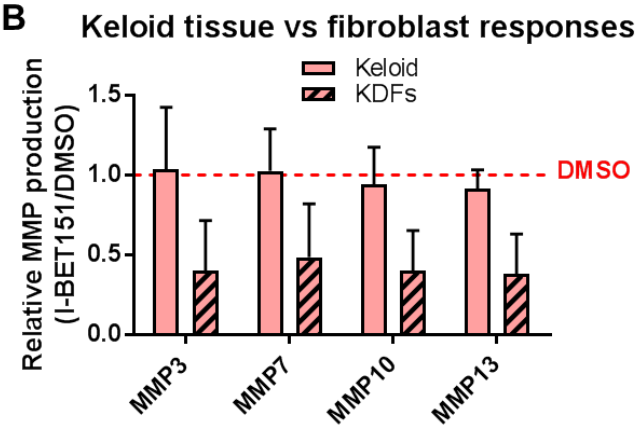
When comparing the Luminex results of the I-BET151 response of *ex vivo* cultures vs fibroblast cultures, an interesting pattern was seen in keloid vs normal skin. MMP3, MMP7, MMP10 and MMP13 showed a consistent downregulation in both normal skin *ex vivo* cultures and NDF cultures (**Figure 4.10A**). However, in the keloid comparison, these proteases were strongly decreased in KDFs, as above, but there was very little change in keloid *ex vivo* cultures (**Figure 4.10B**), suggesting another cell type within keloid scar tissue could be important for releasing these proteases that is non-responsive to I-BET151 inhibition. In addition, these four analytes showed much stronger correlation, by Spearman's correlation analysis, within keloid tissue and not normal skin (**Figure 4.10C & D**).

Given the dogma in the literature that MMP and TIMP interactions occur in a non-biased 1:1 ratio (Tuuttila *et al.*, 1998), a crude measure of protease balance was also calculated by dividing the total quantity of MMPs by the total quantity of TIMPs. This analysis revealed an overall decrease after I-BET151 treatment in 5 out 6 normal skin biopsies and 5 out of 6 keloid biopsies (**Figure 4.11**). Fibroblast cultures also showed a consistent decrease in 'protease activity' with all 6 fibroblast cultures exhibiting a decrease in the I-BET151 treated condition (**Figure 4.11**).

A




B




C

Spearman's R, normal skin

| | MMP7 | MMP10 | MMP13 |
|---|---|---|---|
| MMP3 | 0.543 | 0.829 | 1 |
| MMP7 | | 0.486 | 0.54 |
| MMP10 | | | 0.829 |

Spearman's R, keloid

| | MMP7 | MMP10 | MMP13 |
|---|---|---|---|
| MMP3 | 0.986 | 0.943 | 0.928 |
| MMP7 | | 0.928 | 0.882 |
| MMP10 | | | 0.928 |

***Figure 4.10* - Comparison of *ex vivo* vs fibroblast monoculture production of MMP3, MMP7, MMP10 and MMP13 after I-BET151 treatment.** Luminex data for I-BET151 treated cultures normalised to vehicle control to show mean change in expression after 24 hours I-BET151 or vehicle treatment, in tissue *ex vivo* cultures vs fibroblast cultures in normal skin (**A**) and keloid (**B**), mean +/-SEM, n=3/5. (**C**) Spearman's correlation co-efficient (R) for the level of MMP3, MMP7, MMP10 and MMP13 in control normal skin and keloid supernatants, where underlined = $p<0.05$, n=6.

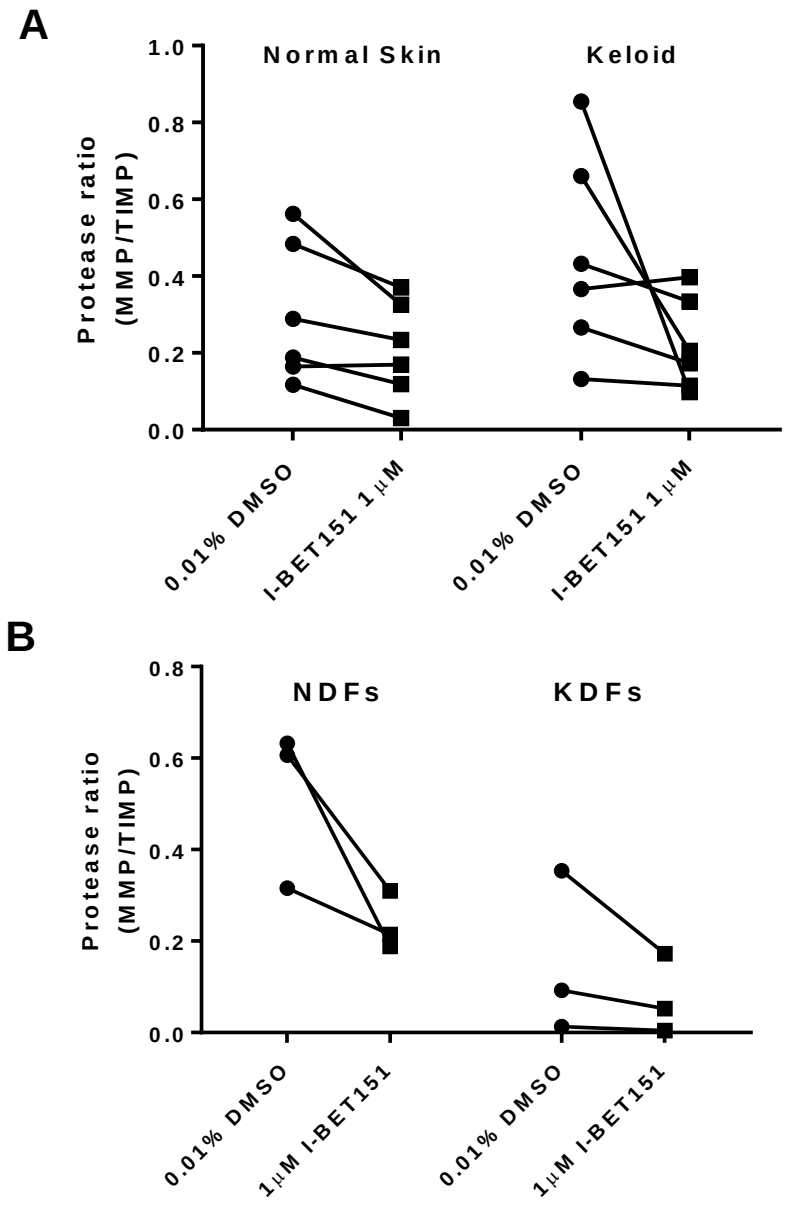



***Figure 4.11*** **- 'Protease ratio' of cultures.** Protease ratio was calculated by dividing total MMP by total TIMP for *ex vivo* supernatants (**A**) and fibroblast culture supernatants (**B**), as measured by Luminex.

### 4.2.4. Decrease in proteolytic activity after BET inhibition

After establishing that the ratio of MMP to TIMPs was decreased with BET inhibition, it was important to identify whether this did indeed cause a reduced proteolytic phenotype in fibroblasts, especially as the Luminex method, in addition to measuring active MMPs, also detected pro-form and TIMP-complexed forms of MMP. In order to answer this question, a zymography technique was used to examine 24 hour serum-free supernatants from 3 NDF and 3 KDF cultures treated with 1µM I-BET151 or 0.01% DMSO. A pan-MMP substrate that produced a colorimetric reaction after cleavage, in order to get an overall picture of MMP activity change, rather than examining particular MMPs in detail.

Absorbance at 412nm was read on a plate reader after the addition of all components to the plate to give a 0 hour baseline reading. The plate was incubated at 37°C for the zymography reaction to take place and absorbance was measured at 6, 24, and 48 hours.

Almost no difference was observed in the water only control throughout the experiment, whereas some increase was seen in the media only control, suggesting there may be some component in the media that has the potential to cause the DTNB to reduce and change colour. Nonetheless, both the NDF and KDF cultures showed protease activity above the media control that increased over time (**Figure 4.12A/B)**. NDFs had higher protease activity over the time course compared to KDFs, with an average of twice as much protease activity at the 24 hour timepoint (0.10 vs 0.04 respectively at 24 hours) (**Figure 4.12B)** which closely matched the protease ratio from the Luminex data, (**Figure 4.11A)**. BET inhibited fibroblast cultures tended to have a decrease on protease activity, especially in NDF cultures that showed a higher level of protease activity to begin with (**Figure 4.12B**).

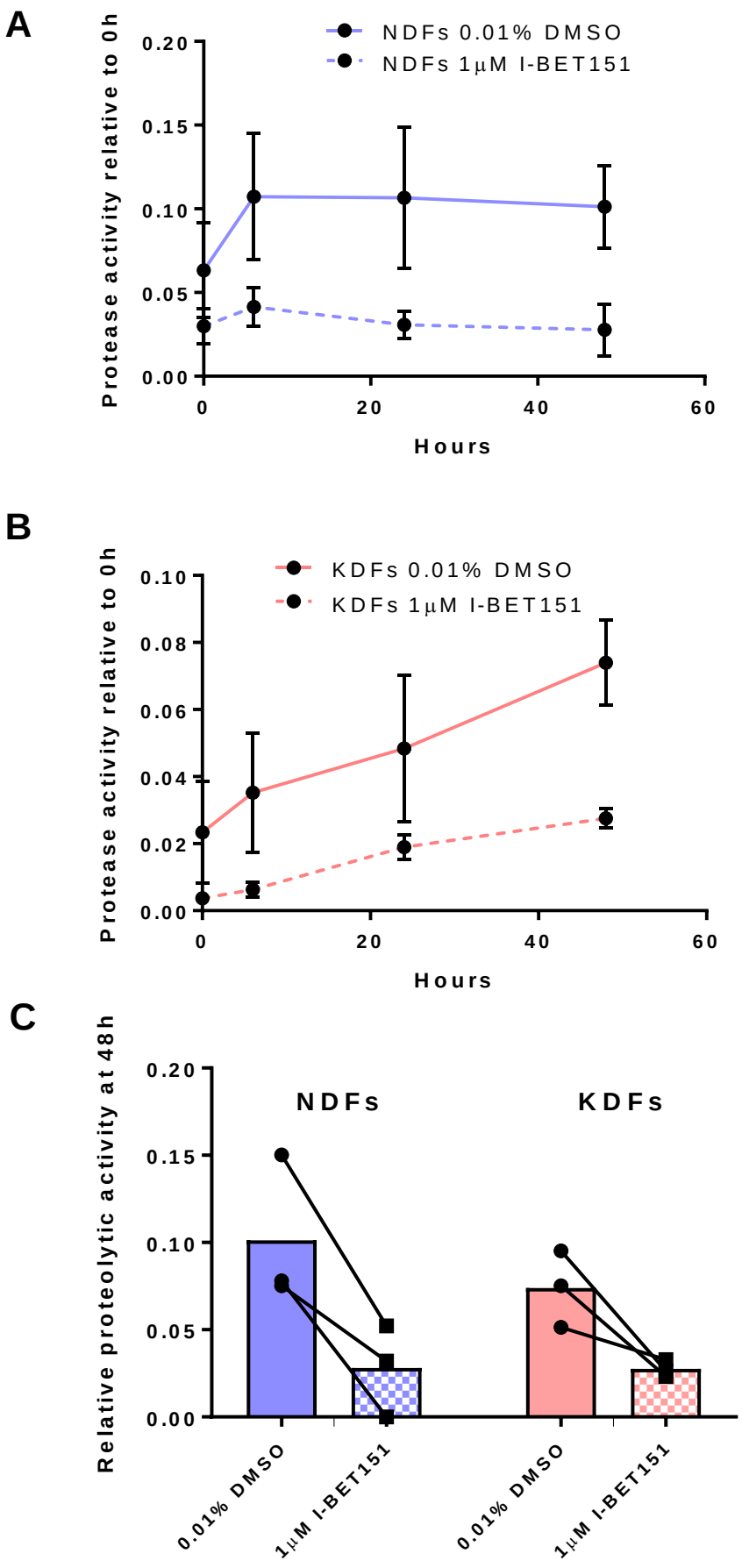



***Figure 4.12* - Proteolytic activity of fibroblasts after I-BET151 treatment** Absorbance at 412nm over time for n=3 NDF (**A**) or KDF (**B**) supernatants treated with 1µM I-BET151 or 0.01% DMSO minus the media control. (**C**) Protease activity at 48 hours minus the media control. Technical triplicates were averaged to plot each biological replicate.

## 4.3. Discussion

Keloid cells have a number of features thought to contribute to the pathology; here the ability of I-BET151 to mediate some of these, including proliferation, collagen gene expression, contraction and protease activity, were investigated.

Firstly, the impact of BET inhibition on fibroblast proliferation was assessed by counting cells after 72 hours of I-BET151 treatment. This experiment showed a significant decrease in proliferation in both NDF and KDFs, although I-BET151 treated wells still had around half the cells of the vehicle control at this timepoint. This was further confirmed with the presence of around 35-45% Ki67 positive cells after 24 hours of I-BET151 treatment, with a percentage decrease compared to the vehicle control of around 35-40%. Remarkably, even at a 10μM dose of I-BET151, approximately 20-30% of fibroblasts were Ki67 positive, indicating that a subset of fibroblasts could be resistance to BET inhibition.

An anti-proliferative effect of BET inhibition has been reported in a range of cell types including cancer cells from prostate cancer (Asangani *et al.*, 2014), myeloma (Chaidos *et al.*, 2014), colon cancer (Hu *et al.*, 2015) and glioblastoma (Ishida *et al.*, 2017), as well as cells from fibrotic conditions such as liver fibrosis (Ding *et al.*, 2015) and lung fibrosis (Tang *et al.*, 2013a). In general, the anti-proliferative effect of BET inhibition is reported to be due to a downregulation of MYC (Delmore *et al.*, 2011). However the role of MYC is not entirely clear in fibroblasts due to its diverse downstream targets (Soucek and Evan, 2011). Although it has been shown that MYC can promote proliferation (Hanson *et al.*, 1994), it has also been shown to increase resistance to apoptosis in low serum conditions (Evan *et al.*, 1992). MYC is reported to be both increased (Hu, Lou and Luo, 2002; Jiao *et al.*, 2016) and decreased in keloid cultures (Cohly *et al.*, 2002) and we have not investigated whether the anti-proliferative effects of BET inhibition in this study are due to an effect on MYC expression.

Resistance to BET inhibition has now been reported in some cancers, where a subset of a heterogeneous tumour cell population subverts the BET-mediated c-Myc transcription that is responsible for driving cell cycling (Settleman, 2016). In triple negative breast cancer this occurs through the non-bromodomain binding of BRD4 to another transcriptional regulator MED1 which can then act to bind and drive transcription of MYC (Shu *et al.*, 2016). Alternatively, in acute myeloid leukaemia, cells can overcome the need for BRD4 interaction completely, instead activating MYC through β-catenin via Wnt signalling (Rathert *et al.*, 2015). The finding that some fibroblasts in our model are still cycling could also indicate that there is

similar heterogeneity in this cell population that is retained in culture, with differing abilities to overcome BET inhibition and continue proliferating.

To understand the reversibility of the BET mediated decrease in proliferation, wash-out experiments were conducted where cells were first treated with I-BET151 or vehicle control for 24 hours followed by washing and removal of the inhibitor and continued culture for up to 48 hours. This experiment illustrated the recovery of active proliferation marked by Ki67 protein levels. The prolonged Ki67 reduction in KDFs, along with the slightly reduced number of KDFs compared to NDFs after 72 hours of I-BET151 treatment, suggests that KDFs may have a slightly higher sensitivity to I-BET151 in terms of the proliferative response. We do not have information regarding binding stability of this drug to the bromodomain of BET proteins, but GSK describe this inhibitor as fully reversible on a timescale measured in minutes. If the increased sensitivity of KDF proliferation in response to I-BET151 holds true, it could reflect possible differences in the factors driving cellular proliferation and potentially varying mechanism of action of this drug which could offer a useful therapeutic advantage *in vivo*.

Interestingly, despite some reports that keloid fibroblasts may be more proliferative (Calderon, Lawrence and Banes, 1996; Satish *et al.*, 2004; Blažić and Brajac, 2006) and especially given the sheer size of keloid scars, there was no observable difference in proliferation between NDFs and KDFs in this project. If anything, there may be a slightly lower proliferation rate in KDFs as evidenced by lower cell counts and Ki-67 percentage in vehicle controls. NDFs may have increased proliferation due to the unnatural environment of *in vitro* culture. In particular, the cells were grown in 10% serum which contains a range of proteins including growth factors that are well established to promote proliferation (Iyer *et al.*, 1999; Pohlers *et al.*, 2009). This promotes the unnaturally proliferative behaviour of fibroblasts in culture. The slightly slower growth rate of KDFs may also be due to missing co-stimulatory factors that promote pathological proliferation *in vivo*. For example, fibroblast proliferation may be driven by other cell types are important in keloid pathology and direct interactions with these cells or their secretions such as basal keratinocytes (Werner, Krieg and Smola, 2007) or immune cells (Wynn, Yugandhar and Clark, 2013). Numerous factors are well recognised to stimulate keloid proliferation, such as IL-6 (Lim *et al.*, 2006), PDGF-BB (Bonner *et al.*, 1990) and TGF-β1 (Clark *et al.*, 1997) which can be produced in an autocrine or paracrine manner.

Conditioned medium from KDFs was not able to promote the proliferation of NDF cultures (Russell *et al.*, 1988). This suggests that what promotes fibroblast proliferation *in vivo* is produced by alternative cellular source, or there is differences in NDFs and KDFs in their ability to sense and trigger signalling after cytokine or growth factor stimulation (Satish *et al.*, 2004). This paper also postulates that KDFs may be more similar to foetal cells and express

growth promoting genes (Russell *et al.*, 1988) although the identity of these genes was not described.

It is also hypothesised that KDF proliferation *in vivo* is exacerbated by a lack of apoptosis; there is less apoptosis in keloid tissue by in situ hybridisation (Sayah *et al.*, 1999) and cultured KDFs are more resistant to ceramide triggered apoptosis than NDFs (Ishihara *et al.*, 2000). Although apoptosis was not directly assessed after BET inhibition in this work, it has been shown in multiple cells types that BET inhibition can induce apoptosis, which again may be mediated through c-myc (Baker *et al.*, 2015; Jostes *et al.*, 2016). Although BET inhibition did not appear to cause notable apoptosis in our homeostatic cultures (no nuclei blebbing or floating cells seen), it would be interesting to measure apoptosis in our cultures after BET inhibition by examination of markers such as caspases following an insult that would promote cell death, with the hypothesis that BET inhibition may abrogate KDF resistance to apoptosis.

We saw some evidence that BET inhibition can reduce collagen transcripts in some donors, in particular in KDFs, especially COL3A1. However, at the protein level there was no change in collagen I secretion in supernatants and unexpectedly KDFs showed decreased collagen I compared to NDFs. This may be because most collagen that is secreted is not soluble and is instead deposited onto the cell culture plate, or that the length of treatment is not long enough to see a substantial difference at the protein level. Further analysis could be done to look at cell lysates that are scraped directly from plates to capture this deposited collagen. Reduced collagen has been observed by others at the RNA and protein level in renal interstitial fibroblasts after I-BET151 treatment although the concentration was increased to 5μM to see a strong effect (Xiong *et al.*, 2016). Decreased COL1A1 transcript has also been reported after BET inhibition in TGF-β1 treated lung fibroblasts (Tang *et al*. 2013b). Interestingly, a study in pancreatic stellate cells saw a decrease in collagen RNA and protein after treatment with I-BET151 or siRNA against BRD4, however siRNAs against BRD2 or BRD3 increased expression of matrix proteins (Kumar *et al.*, 2017).

We next examined the effect of I-BET151 on cellular contractility using a collagen gel contraction model. We observed a statistically significant decrease in contraction after 72 hours of I-BET151 treatment. Increased contractility contributes to other fibrotic conditions such as scleroderma (Chen *et al.*, 2005) and scarring trachoma (Kechagia *et al.*, 2016) and could have relevance in keloids although the evidence for this is scant. Undoubtedly, contractile forces are important during wound repair (Hinz, 2007) and persistence of this tension can exacerbate ‘normal’ scarring (Hinz *et al.*, 2012). Keloid scars are often characteristically taut and stretched across areas of skin tension and are more likely to form at sites of high tension such as the sternum or shoulders (Gauglitz *et al.*, 2011). Reducing tension in this context may give benefit in reducing mechanosensitive pathways, such as Rho and Erk signalling (Kenny and Connelly,

2014), and reducing clinical pain (Bordoni and Zanier, 2013). Stretching tension at wound edges has also been associated with the outgrowth of keloid tissue and therefore have seeded the use of compression dressings to prevent keloid growth (Ogawa, 2017). Clinical reports of the advantageous effects of Botox treatment of keloid scars may be attributable to a reduction in tension (Robinson, Khadim and Khan, 2013)

A plethora of cell behaviour and expression profiles have been linked with changes in tension. In particular, tension has been shown to increase fibroblast proliferation (Webb *et al.*, 2006), decrease apoptosis (Aarabi *et al.*, 2007) and increase myofibroblast differentiation (Hinz *et al.*, 2001). One particularly elegant study seeded NDFs and KDFs into a 3D model of *in vivo* tension, and showed increased proliferation, especially in KDFs, and increased expression of Hsp27, PAI-2 and α2β1 integrin which appeared to mediate the proliferative response as knockdown of these factors prevented the tension mediated increase in proliferation (Suarez *et al.*, 2014). Integrins are extremely important for mechano-sensing, allowing cells to bind to a range of matrix proteins and trigger downstream signalling leading to changes in cell behaviour (Kenny and Connelly, 2014); therefore it is likely relevant that keloid cells have been shown to express increased α2β1 integrin compared to cells from both hypertrophic scar and normal skin (Szulgit *et al.*, 2002). It would therefore be insightful to examine the expression of machinery for the adhesion to the collagen matrix which could affect the contractile ability of fibroblasts in both NDFs and KDFs with and without I-BET151 treatment. It is possible that BET inhibition could reduce the expression of this machinery (e.g. α2β1 integrin) causing less adhesion to matrix and therefore less contraction. Indeed, scratch closure migration assays showed a trend for increased fibroblast migration after BET inhibition (data not shown) which may suggest a loss of adhesion molecules (Tamariz and Grinnell, 2002).

The non-cellular environment could also impact on contractility. *In vivo* contractility can induce a positive feedback loop through the activation of latent, matrix-bound TGF-β1, which can go on to cause further myofibroblast differentiation and contraction (Wipff *et al.*, 2007). Therefore contractility is a relevant and important cell behaviour to target, and it may be worth exploring the ability of I-BET151 to reduce contraction mediated by relevant growth factors (e.g. TGF-β1) or co-cultured with macrophages, which have been shown to accelerate scarring trachoma fibroblast contraction (Kechagia *et al.*, 2016).

Some KDFs had a higher contractile ability compared to NDFs with a reduced gel size in the vehicle controls. Although this was not statistically significant, this *in vitro* culture is anticipated to under represent the *in vivo* contractility of the cells which can be influenced by immune cell signals that are likely to be more abundant in the keloid environment compared to normal skin and scar (Bagabir *et al.*, 2012a). It has been shown that platelets (Zagai *et al*. 2003) and eosinophils (Zagai *et al.*, 2004) can induce collagen gel contraction by lung fibroblasts.

Macrophages may also contribute to the contractile phenotype, although depending on the model used it has been shown that macrophages can both exacerbate (Zhu *et al.*, 2001; Kechagia *et al.*, 2016) or inhibit fibroblast contraction in the resolving stages of wound healing (Newton *et al.*, 2004).

We found that both NDFs and KDFs expressed the myofibroblast, contractile marker α-SMA at the mRNA level and this was decreased after 48 hours of I-BET151 treatment, suggesting a decrease in the myofibroblast phenotype of these cells. However, cells extracted from the collagen gels did not show any consistent change in α-SMA protein levels following I-BET151 treatment (data not shown). A recent study in the field of liver fibrosis found a reduction in α-SMA protein in hepatic stellate cells after JQ1 treatment (Ding *et al.*, 2015). In further *in vivo* work they demonstrated a therapeutic benefit of BET inhibition in a model of liver fibrosis and attributed this to a decrease in the myofibroblast phenotype, although speculatively link this specifically to Brd4 despite using the BET family inhibitor JQ1 (Ding *et al.*, 2015). Even more recently, I-BET151 has been shown to reduce both basal and TGF-β1 induced α-SMA in cultured renal interstitial fibroblasts (Xiong *et al.*, 2016). They also showed more convincingly that this may be as a result of BRD4 in particular, since the same effect was seen with BRD4 siRNA (Xiong *et al.*, 2016).

One IHC study suggested that only 45% of 40 keloid samples showed α-SMA expression, compared to 70% of the 40 hypertrophic scars examined (Lee *et al.*, 2004) and this may simply reflect the mature stage of keloid development when histology was carried out. It is likely that keloid tissue has been growing for many months, if not years, at the point it is surgically excised and available for this analysis. In our sample collection, the mean age of scar was 6 years and the median was 5 years. Potentially, α-SMA expression was expressed early in keloid formation but lost in a more mature scar.

Another study indicated increased α-SMA in hypertrophic scars compared to keloid scars, although this difference was lost upon *in vitro* culturing (Ehrlich *et al.*, 1994). This is evidence that *in vitro* culturing in a monolayer on plastic affects α-SMA expression in NDFs and KDFs, as has been seen in our western blot analysis that shows no difference in expression between the two cell types (data not shown) which is not unexpected. We postulate that this may be due to both a possible decrease in α-SMA expression in KDFs and a possible increase in expression in NDFs due to the artificial culturing environment.

In addition to the debatable absence or presence of α-SMA in keloid pathology, the expression of this protein has also been reported to be dispensable for contraction *in vitro* in other disease contexts such as scarring trachoma (Kechagia *et al.*, 2016). Therefore the mechanism of collagen gel contraction by these dermal fibroblasts, as well as the mechanism by which I-BET151 reduces contraction may not be entirely dependent on α-SMA. Instead, other actin-

myosin networks could be important in fibroblast contraction (Hinz, 2007) and influenced by BET inhibition, the examination of which was outside of the scope of this project.

It is possible that the inhibitory effect of I-BET151 treatment on collagen gel contraction was as a result of reduced proliferation; proliferation of cells within the gels was not directly measured, although the reduction of α-SMA suggests a true anti-contractile effect of BET inhibition. Experiments to address this question further could include: seeding increasing amounts of fibroblasts in collagen gels to see if there is a linear correlation between cell number and contraction of the gel; running the contraction assay with the addition of the anti-proliferative agent such as mitomycin C to identify if I-BET151 can still decrease contraction independent of proliferation; and a collagen contraction assay seeded with a higher number of fibroblasts but measured over a shorter time span to eliminate proliferation as a variable. However, it has been previously shown that scarring trachoma fibroblasts have an intrinsically higher level of contractility than control fibroblasts in a collagen gel model, which was explicitly shown not to be dependent on cell proliferation or survival, although the mechanism for this increased contraction is unknown (Kechagia *et al.*, 2016).

Importantly, there is no evidence that the collagen gels were being degraded rather than contracted in our model, as a small number of gels were weighed on a fine balance and did not showed any difference in mass between vehicle control treated (i.e. contracted) and I-BET151 treated (i.e. larger) gels (data not shown). Despite no evidence for degradation, MMPs have been shown to be important for collagen gel contraction by fibroblasts (Daniels *et al.*, 2003) and we have also seen decreases in MMP production after I-BET151 treatment which could provide a mechanism for the reduced contractility we have observed and will be discussed next.

When keloid tissue *ex vivo* models appeared to degrade the surrounding collagen gel, it was postulated that the cells within the tissue were producing proteases to cause this effect. Anecdotally, this response was more common in keloid scar than normal skin. We have two main hypotheses; firstly that it is due to an exacerbated response to the wounding inflicted by the biopsy punch or that it is be due to the proteolytic nature of keloid edge tissue. Upon biopsying, keloid cells may be inappropriately activated and upregulate the production of proteases causing the excessive degradation of the collagen gels we have observed. The abnormal ECM environment of pure collagen I itself, with different physical properties, may also induce specific protease production from cells. Unpublished non-cellular proteome data from the lab give strong evidence for an altered and unusual ECM content in keloids that may induce a differential protease production from resident cells even after removal from the environment. The variation in the basal level of protease production and activity in our models could also be reflective of different biopsy locations within the keloid. There has been almost no literature attention on spatial location of MMP expression as most approaches have studied

MMPs at mRNA or protein level in whole tissue without specifics of the location of biopsying such as the fibrotic core vs the actively growing margin. However, unpublished IHC work has shown spatial variation with increased expression of a number of MMPs at the edge region of keloid tissue, compared to very little expression within the core of the lesion (personal communications, Dr Zoe Drymoussi, QMUL). One study also showed increased MMP2 expression in keloid scar at the edge of collagen bundles which could also facilitate this invasive, growing phenotype of the keloid edge region (Imaizumi *et al.*, 2009). The edge region of the keloid is actively growing and has been described as locally 'invasive' (Muir, 1990; Ogawa, 2017) with the clinical definition of keloid scarring being that the scar expands outside the origin wound margin. Increased proteolytic activity, through increased proteases such as MMPs and/or decreased endogenous inhibitors such as TIMPs at this growing wound edge may serve as invasive and therefore pathological in terms of keloid outgrowth.

Based on the hypothesis that the collagen was being degraded, additional experiments were undertaken to substantiate this interpretation. 24 hour supernatants from these *ex vivo* models, as well as from fibroblast monolayers, treated with I-BET151 or vehicle control were analysed by Luminex for levels of MMPs and TIMPs.

The Luminex analysis confirmed heterogeneity in patient samples, both in whole tissue and in isolated fibroblasts cultures. Nonetheless, in terms of the basal level of expression, vehicle control KDFs showed lower levels of most MMPs and increased levels of TIMP-1, the most highly expressed inhibitor of MMP in these fibroblasts, compared to NDFs which at face value could contribute to excess matrix accumulation. Indeed when the proteolytic ratio was calculated from this data, by dividing total MMP by total TIMP, this confirmed slightly increased proteolytic capacity in NDFs versus KDFs, whilst conversely keloid tissue generally showed an increased proteolytic capacity compared to normal skin.

The levels of MMPs measured in fibroblast monolayers may not be fully representative of their *in vivo* phenotype due to the absence of a secretory signals such as PDGF-BB (Bauer *et al.*, 1985), TGF-β1 (Edwards *et al.*, 1987) and IL-6 (Dasu *et al.*, 2003), or direct interaction to other cell types that could be present *in vivo* that may induce protease expression in fibroblasts. For example it is also thought that mast cells can interact directly with fibroblasts to induce MMP-9 production through Erk signalling (Abel and Vliagoftis, 2008) which have also been reported as present in keloid scars (Bagabir *et al*. 2012a).

It would therefore be interesting to treat cells with such wound-associated signals to explore NDF and KDF capability to produce MMPs upon stimulation and the capability of I-BET151 to mediate this. Incubation of fibroblasts with pro-inflammatory macrophages is reported to increase production of MMP-1, MMP-2, MMP-3, MMP-14 and TIMP-1 with an overall 3 fold increase in proteolytic activity (Ploeger *et al.*, 2013). Notably this effect was mitigated by the

addition of conditioned media from M2 macrophages (Ploeger *et al.*, 2013) suggesting that the balance of inflammation in the keloid environment may affect MMP production in fibroblasts to cause the potentially elevated levels of protease activity we have seen in whole keloid tissue.

Indeed within the *ex vivo* culture models there are a range of cell types that can contribute to MMP production such as immune cells which are known to produce MMP8, MMP9 and MMP14 for example (Rohani and Parks, 2015). It is true that the comparison of normal skin and keloid tissue biopsies is problematic as keloid tissue is likely to be more cellular including a strong immune cell presence and have a greater depth with a thicker epidermis. However, this does make the equal or even slightly lower level of TIMP in keloid tissue vs normal skin tissue particularly striking.

Despite heterogeneity amongst the patient tissue and cells, I-BET151 generally decreased the production of MMPs with no clear effect of TIMPs in all cultures. The most striking effect was the substantial decrease in proteolytic ratio after I-BET151 treatment, in both normal skin and keloid scar, and in NDFs and KDFs. In addition, the zymography experiment, despite showing heterogeneity between patient samples, also showed a decrease in proteolytic activity of NDFs and KDFs after I-BET151 treatment.

There have been some limited observations of MMP changes after BET inhibition in the literature including one publication that showed a reduction in TNFα and IL-1β induced production of MMP1 and MMP3 in synovial fibroblasts (Klein *et al.*, 2014). In this case, MMP activity is pathological as it contributes to the matrix destruction characteristic of RA. This paper eludes to these MMP differences being as a secondary result of the anti-inflammatory nature of BET inhibition. However, another study has shown a reduction in MMP2 and MMP9 after *BRD4* knock down, which was a result of reduced signalling through the Sonic hedgehog pathway (Wang *et al.*, 2015). Furthermore, a phosphorylation dependent activity of Brd4 has been shown to directly induce MMP9 expression through chromatin recruitment of AP-1 and NF-κB (Wu *et al.*, 2016). It is possible that the downregulation of MMP we have seen is due to a decrease in AP-1 activity, as RA synoviocytes treated with JQ1 show a strong downregulation of the AP-1 subunit c-Jun (Zhang *et al*. 2015) and AP-1 is a well-established, common regulator for almost MMPs (Benbow and Brinckerhoff, 1997).

The decrease in proteolytic activity seen after I-BET151 in our models may also have relevance in other diseases, as although an increase in MMP:TIMP ratio has been associated with scar free healing in studies in mammalian foetuses (Dang *et al.*, 2003), it has also been associated with oncogenic tumour growth and metastasis (Deryugina and Quigley, 2006; Shay, Lynch and Fingleton, 2015) hence the historical development of MMP inhibitors for cancer (Deryugina and Quigley, 2006).

Differential responses to I-BET151 were seen in whole tissue versus fibroblast cultures, with an interesting difference between normal skin and keloid scar. MMP3, MMP7, MMP10 and MMP13 were regulated by I-BET151 in a similar manner in both normal skin tissue and NDFs, whereas these MMPs were all downregulated in KDFs whilst being relatively unaffected in keloid tissue. As has been previously discussed, keloid tissue may have a different cellular composition, especially in terms of immune cells and keratinocytes, which are capable of producing a range of MMPs (Kähäri and Saarialho-Kere, 1997). It is known that the effects of BET inhibition are highly cell specific (personal communications, GSK) and so the variety of cellular sources for MMP may explain this differential response to BET inhibition. Interestingly, these four MMPs had a very strong positive correlation in keloid tissue unlike in normal skin suggesting they may be produced by a common cell type abundant in keloids.

There is a large volume of conflicting literature surrounding the presence or absence of MMPs and their subsequent role in keloid aetiology. This leaves a confusing picture of MMP contribution to keloid scar initiation and growth. However, given the hypothesis that MMP activity may be increased at the actively growing edge of the keloid lesion, whilst decreased in the fibrotic core, the ultimate goal of an epigenetic approach in this context would be to raise proteolytic activity in the fibrotic core of the keloid whilst decreasing proteolytic activity at the growing edge. This ‘tailored’ approach would be superior to the widely studied MMP inhibitors, many of which have failed clinical trials in cancer, due to off-target effects of pan-MMP reduction which may due to the impact on the ‘good’ and ‘bad’ effects of MMP activity (Fingleton, 2008).

A key limitation of this project was the lack of stratification of samples between the keloid core and keloid edge regions which are likely to have distinct characteristics, such as increased proteolytic activity, cellular metabolism, or proliferation at the invasive, actively growing edge, compared to the hypoxic, matrix dense keloid core. In future work, it would be pertinent to increase sample stratification to address this, and to further understand the effect of BET inhibitors on these distinct regions to better evaluate its value as a potential keloid therapeutic *in vivo*.

# Chapter 5: Investigating the effect of BET protein inhibition on differential IL-6 signalling in keloid scars

## 5.1. Introduction

In resting human skin, IL-6 is generally restricted to the epidermal layer (Romero and Pincus, 1992). Upon stimulation or wounding, IL-6 can be produced as part of the acute phase response (Gruys *et al.*, 2005; Murphy, Travers and Walport, 2008). It is thought that keratinocytes produce much of this IL-6, but other key players include fibroblasts and immune cells such as macrophages and mast cells (Paquet and Pierard, 1996). Additionally, it has more recently been found that melanocytes, which were previously thought to be immunologically 'inert', are also capable of producing IL-6 *in vitro* when stimulated with IL-1β (Tam and Stępień, 2011).

The importance of IL-6 in wound repair is shown by the substantial delay of wound healing in IL-6 deficient mice, which show slower re-epithelisation, less granulation tissue formation and less inflammatory influx (Luster, 2000). This effect was reversed with the addition of recombinant IL-6. The same group later showed that the addition of recombinant IL-6 could also rescue delayed healing in immunosuppressed mice (Gallucci *et al.*, 2001).

Despite its importance in normal wound healing, high levels of IL-6 has been associated with fibrosis by propagating a chronic inflammatory state (Fielding *et al.*, 2014). This suggests the precise temporal regulation of IL-6 in healing wounds is important for successful wound repair without the development of fibrosis or scarring.

In the early 1990s it was shown that IL-6 stimulation of human dermal fibroblast cultures could induce the production of collagen (Duncan and Berman, 1991). Although the authors postulated that this could be driving pathology in some skin diseases, they stated there was currently no evidence for overzealous IL-6 production in diseased fibroblasts and these cells are responding in a paracrine manner to IL-6 produced by another cellular source.

Evidence for increased IL-6 in keloids compared to normal skin and normal scars has accumulated in the past two decades (Xue, McCauley and Zhang, 2000; Ghazizadeh *et al.*, 2007; Zhang *et al.*, 2009). There is only a single paper suggesting a chronic presence of system IL-6 in keloid patients, with elevated IL-6 in patient PBMCs compared to healthy controls (McCauley *et al*. 1992). This is not surprising given that keloid scarring tends to be an anatomically isolated clinical issue, with no general inflammatory phenotype. It is likely that IL-6 is locally dysregulated, as has been proposed in conditions including systemic sclerosis

(Paquet and Pierard, 1996). There are no reports for wounding experiments in IL-6 overexpression mice, however at rest these mice are not thought to have substantial skin phenotypes, with the exception of a thickened stratum corneum layer (Turksen *et al.* 1992). It is therefore difficult to predict the effect of elevated IL-6 in the scenario of keloid scarring, although it has been shown that inhibition of IL6r in KDFs could reduce ECM production and proliferation (Ghazizadeh *et al.*, 2007).

The current literature in the keloid field, plus evidence from other disease fields including systemic sclerosis (Khan *et al.*, 2012), suggests that IL-6 may have importance in keloid biology, specifically driving fibroblast behaviour including collagen production and proliferation (Zhang *et al.*, 2005; Ghazizadeh *et al.*, 2007; Yu *et al.*, 2014). Although typically thought to exert its effects through Stat3, other downstream pathways including those driven by Erk and Akt could also have a part to play in cellular responses to IL-6 (Wegiel *et al.*, 2008; Rodriguez-Berriguete *et al.*, 2010). Given that IL-6 is a cytokine that appears to be consistently upregulated in keloids in several studies, it has become an increasingly attractive therapeutic target, in spite of limited evidence about its effects.

BET protein inhibition has been demonstrated to decrease NF-κB mediated transcription, including IL-6, which is thought to be mediated mainly through BRD2 in melanoma cells (Gallagher *et al.*, 2014) and BRD4 in lung epithelial cancer cells though direct interaction with RelA . Additionally, it has been found that BET proteins can drive transcription of IL-6 by specifically regulating the binding of CBP to the IL-6 promoter region and this can be blocked with BET inhibition (Belkina, Nikolajczyk and Denis, 2013; Barrett *et al.*, 2014).

This chapter therefore investigates IL-6 expression and activity in keloids and the effect of BET inhibition on this with the following key objectives:

1. Confirm elevated IL-6 production in keloid tissue and keloid dermal fibroblasts as reported in the literature
2. Investigate the ability of NDFs and KDFs to respond to IL-6
3. Explore the effect of BET inhibition on IL-6 production and downstream signalling in dermal fibroblasts

Experiments were conducted in both 10% serum, as per usual tissue culturing conditions and serum free conditions.

## 5.2. Results

### 5.2.1. IL-6 production is increased in both keloid tissue and keloid dermal fibroblasts

The first part of this study aimed to confirm whether keloids have elevated IL-6 production, as has been previously described in the literature (Xue, McCauley and Zhang, 2000; Uitto, 2007; Zhang *et al.*, 2009). Supernatants were taken after 24 hours of culture of fibroblast monolayers. Supernatants were also taken after 24 hours of culture of tissue *ex vivo* models as used for the previous Luminex analysis. An IL-6 DuoSet ELISA kit from R&D Biosystems was used to measure levels of IL-6 in these supernatants plated neat and at 1:10 dilution in reagent diluent (1% BSA in PBS). Absorbance results at 420nm for each sample were compared to the standard curve.

This analysis did confirm elevated levels of IL-6 production in keloid tissue *ex vivo* cultures compared to normal skin (**Figure 5.1A**), as well as significantly increased IL-6 production in KDF cultures compared to NDF cultures (**Figure 5.1B**).

As culturing cells with serum may induce the production of cytokines including IL-6 (Chang *et al.*, 2004), IL-6 production by fibroblast monolayers in serum free conditions was also analysed in a single NDF and KDF line at 24 hours (**Figure 5.1C**). 72 hour supernatants were also analysed as these cultures were expected to produce much lower levels of cytokines. Indeed, it was found that fibroblasts in serum free conditions around 10 fold less IL-6 than in 10% serum, but cytokine production was still markedly higher in the KDF line. IL-6 was below the level of detection in the NDF line until 72 hours of culture, whereas 3.2ng/ml of IL-6 was detected in the KDF line at 24 hours.

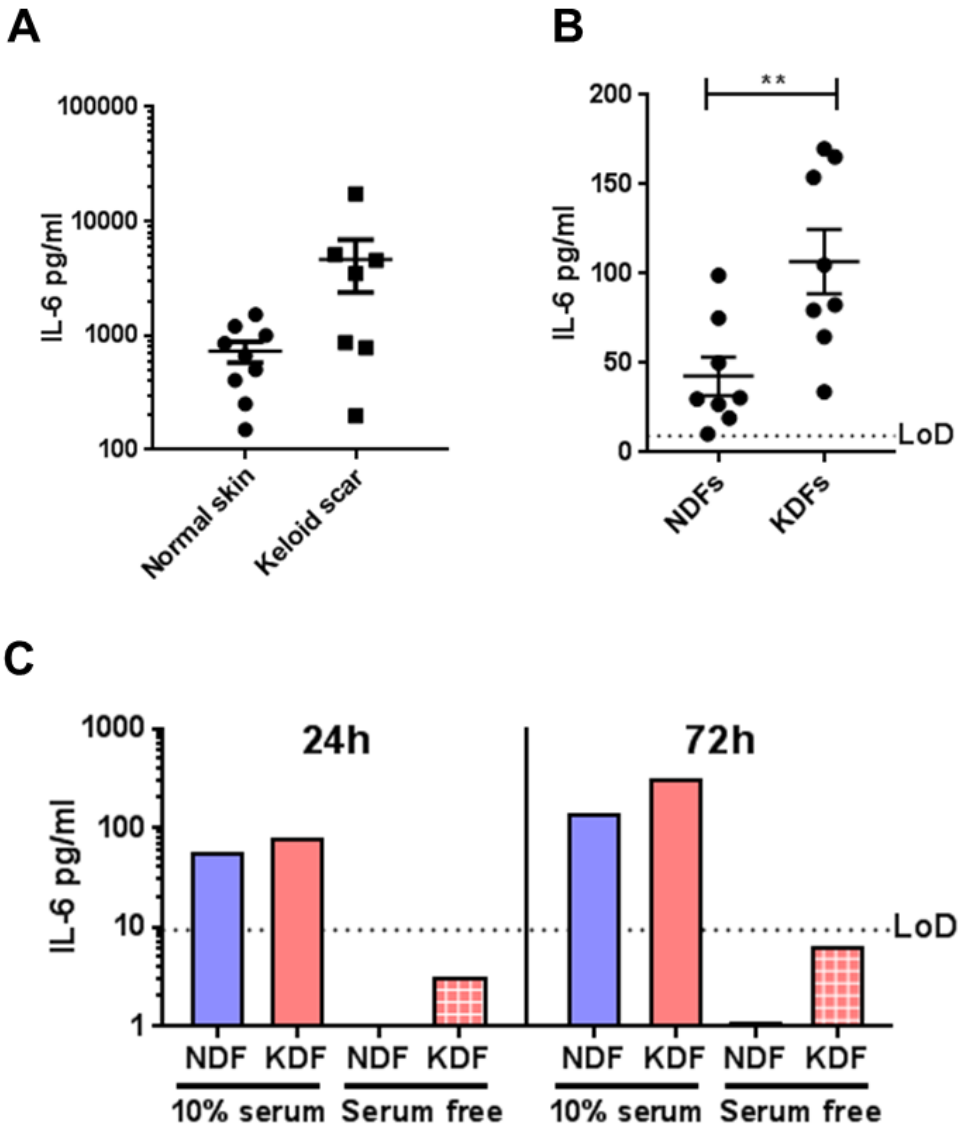



***Figure 5.1* - Elevated IL-6 in keloid tissue and fibroblast cultures.** IL-6 ELISA of 24 hour supernatants from n=7-9 normal skin and keloid scar *ex vivo* cultures (**A**) and n=8 NDF and KDF cultures in 10% serum (**B**) ** = p<0.005, Mann-Whitney test, mean +/- SEM) and of 24 hour and 72 hour supernatants from a NDF and a KDF culture in both 10% serum and serum free conditions (**C**), where IL-6 production in serum free NDF culture was undetectable at 24 hours. Data is plotted on a logarithmic scale to visualise the low level of expression in serum free cultures.

### 5.2.2. In serum free conditions, KDFs have elevated basal pStat3, reduced by IL-6r blockade, and elevated pStat3 induction after IL-6 stimulation

To examine the level of signalling downstream of IL-6 in three possible downstream pathways that may mediate the cytokine response (Stat3, Erk and Akt), cells were cultured in both 10% serum and serum free conditions before ligand-stimulated activation of each pathway was analysed by western blot. Briefly, three NDF and KDF lines were cultured in 10% serum and serum free conditions for at 24 hours before treating with 10ng/ml human recombinant IL-6 for 30 minutes and protein lysis. Blots were probed for the phosphorylated form of each signalling molecule as readouts of the three potential downstream pathways as well as GAPDH as a loading control. Blots were then stripped with a mild stripping buffer for 20 minutes before washing and re-probing for the total form of each signalling molecule.

Then, to explore whether IL-6 was responsible for driving elevated basal signalling of these three pathways in KDFs, Tocilizumab was used to block IL-6 signalling in fibroblast cultures. Tocilizumab is a blocking antibody which can target and block IL-6r in both membrane bound and soluble form and is used in the clinic for inflammatory arthritis including Rheumatoid Arthritis (Jones and Ding, 2010). NDFs and KDFs in 10% serum and serum free conditions were treated for 72 hours with increasing concentrations of 1, 10 and 100µg/ml of Tocilizumab, based on literature review (Suzuki *et al.*, 2010; Oguro *et al.*, 2013; Sakkas, 2016) and personal communications with colleagues who frequently use this antibody.

Firstly, Stat3 signalling was examined. In 10% serum conditions, it was observed that IL-6 stimulation was able to increase pStat3 levels in all NDFs and 2 out of 3 KDFs tested. However, there was no observable difference between NDFs and KDFs in 10% serum conditions, in terms of basal or IL-6 stimulated levels of pStat3 (**Figure 5.2**). The removal of serum from the system revealed more discrete differences between the NDFs and KDFs tested; all 3 KDFs showed heightened basal pStat3 with no observable pStat3 band in NDFs in this condition even with a 20 minute blot exposure. In serum free conditions, IL-6 stimulation caused a modest induction of pStat3 but generally showed a greater induction in KDFs. Blockade of IL-6r by 72 hours of Tocilizumab treatment caused some reduction in basal pStat3 levels at increasing doses (**Figure 5.3**) although this was most striking in KDFs in serum free culture (**Figure 5.3B)**. However, detectable levels of pStat3 were still observed even with Tocilizumab treatment at a concentration of 100µg/ml.

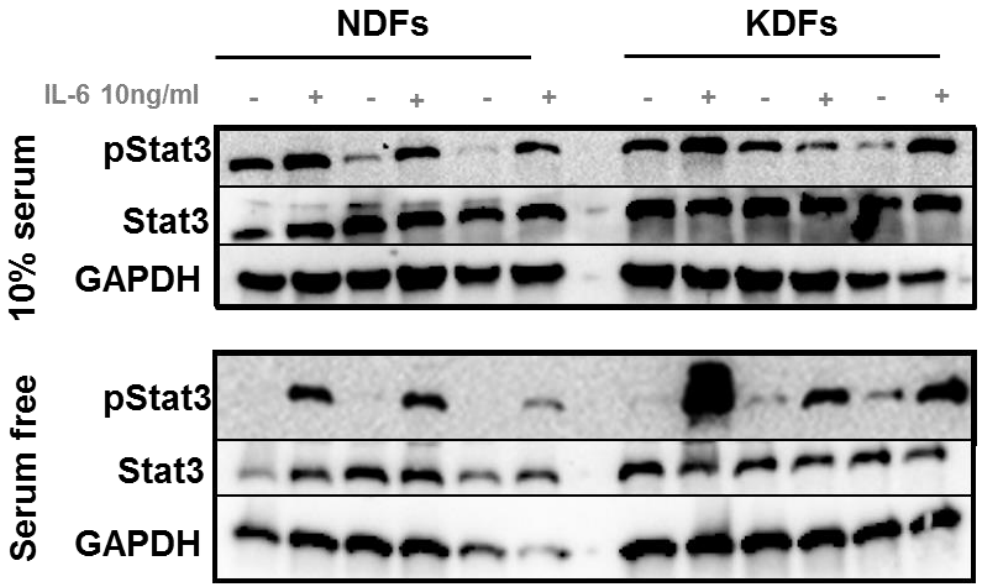



***Figure 5.2* - Western blot showing basal Stat3 signalling and after acute IL-6 simulation in dermal fibroblasts**. n=3 NDF and KDF cultured in 10% serum and serum free conditions were lysed after a 30 minute stimulation with 10ng/ml IL-6. Blots were probed for pStat3, Stat3 and GAPDH to examine the level of Stat3 activation basally and after IL-6 stimulation.

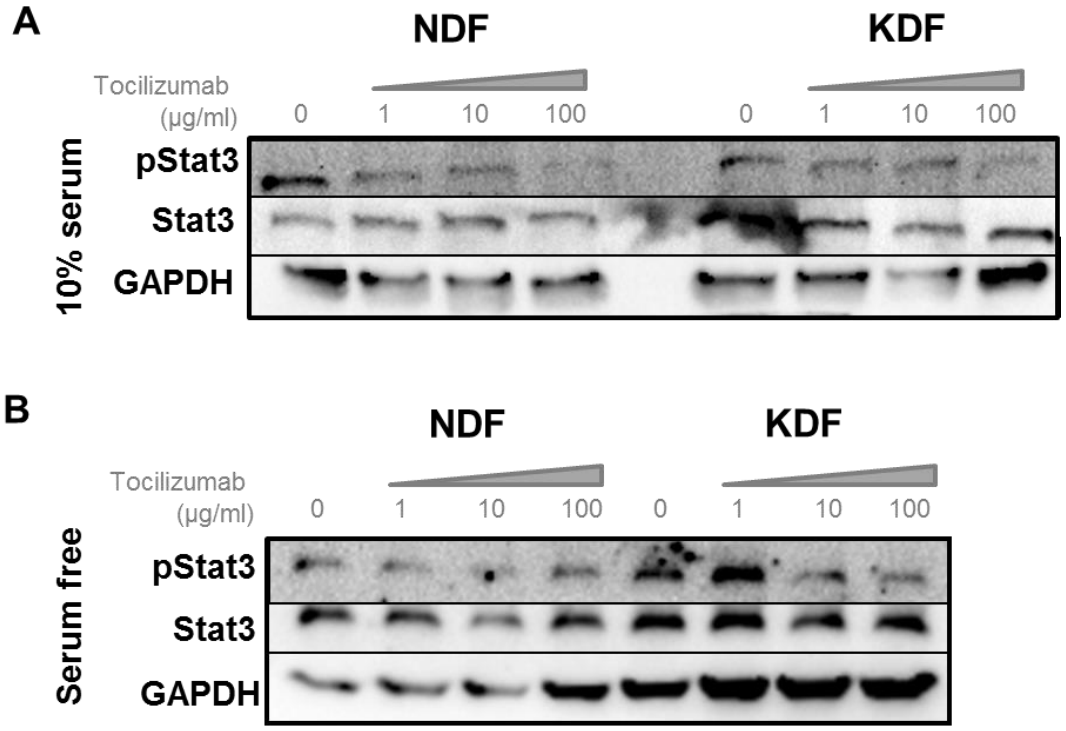



***Figure 5.3* - IL-6r blockade reduces basal pStat3 in NDFs and KDFs.** Western blot of NDF and KDF cultures in 10% serum (**A**) and serum free conditions (**B**) treated for 72 hours with increasing concentrations of the IL-6r blocking antibody Tociluzumab. Blots were probed for pStat3, Stat3 and GAPDH in order to identify the contribution of IL-6 in these cultures for driving basal pStat3 activity.

### 5.2.3. Only KDFs in serum free conditions activate pErk following IL-6 stimulation and decrease pErk after IL-6r blockade

Next, Erk signalling was examined, stripping of the pErk1/2 was deemed unsatisfactory, so GAPDH alone was used as a control for these blots (**Figure 5.4**). In 10% serum conditions, although there were higher basal levels of pErk in 2 out of 3 KDFs compared to NDFs, phosphorylation was not affected by IL-6 stimulation in either cell type (**Figure 5.4**). Whereas in serum free conditions, there was no difference in basal pErk in KDFs compared to NDFs, but KDFs had some induction after IL-6 stimulation which wasn't seen in NDFs (**Figure 5.4**).

Again, the effect of blocking IL-6 driven Erk signalling was examined in Tocilizumab treated cells. In 10% serum conditions, there was no effect of Tocilizumab in NDF or KDF, although basal levels were fairly low in these cultures (**Figure 5.5A**). However, in serum free conditions, Tocilizumab did reduce pErk in the KDF line at the lowest dose with no concentration response observed (**Figure 5.5B**).

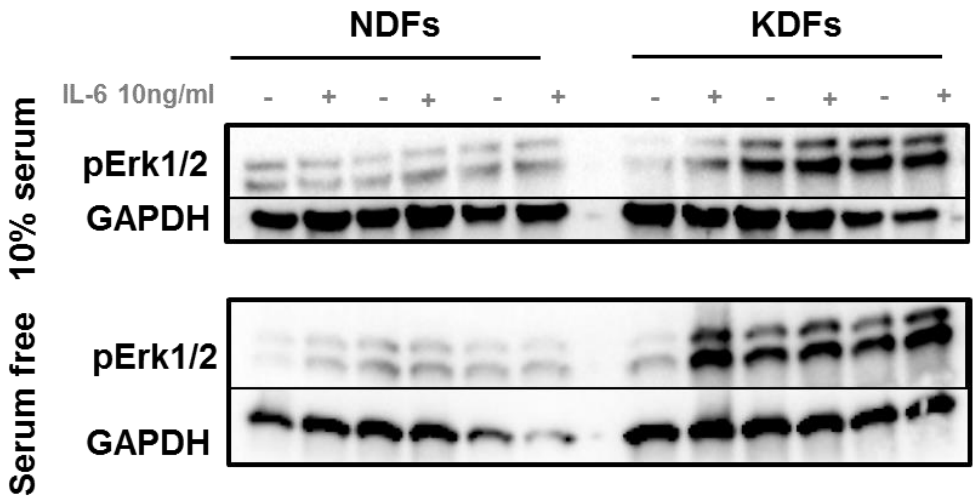



**Figure 5.4 - Western blot showing pErk signalling at the basal level and after acute IL-6 stimulation in dermal fibroblasts**. n=3 NDF and KDF cultured in 10% serum and serum free conditions were lysed after a 30 minute stimulation with 10ng/ml IL-6. Blots were probed for pErk and GAPDH to examine the level of Erk activation basally and after IL-6 stimulation.

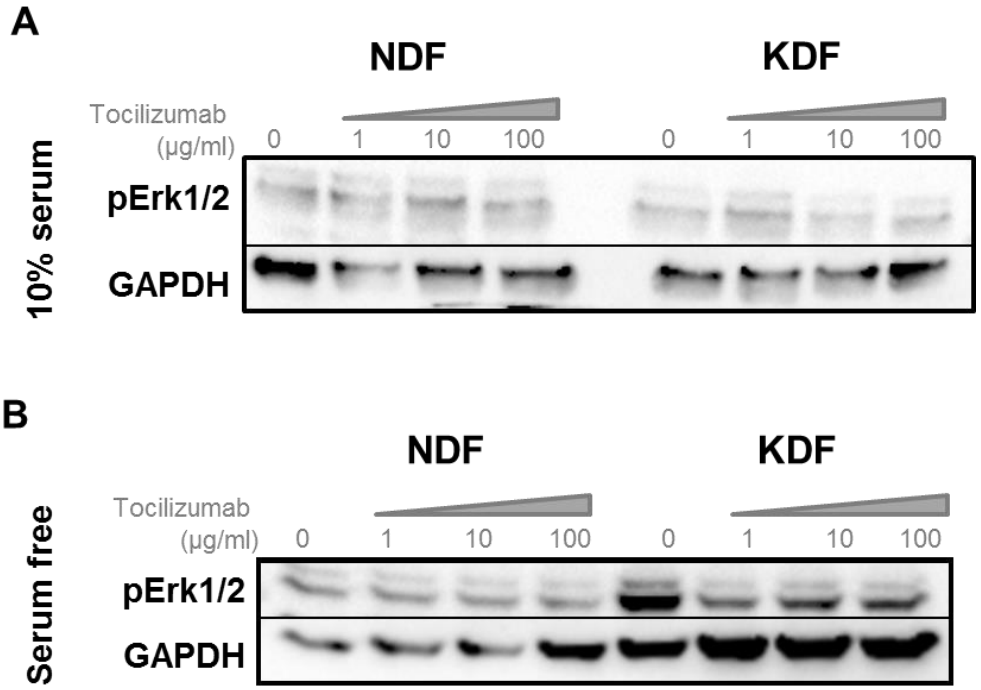



***Figure 5.5* - Consequence of IL-6r blockade on basal pErk in NDFs and KDFs.** Western blot of NDF and KDF cultures in 10% serum (**A**) and serum free conditions (**B**) treated for 72 hours with increasing concentrations of the IL-6r blocking antibody Tociluzumab. Blots were probed for pErk1/2 and GAPDH in order to identify the contribution of IL-6 in these cultures for driving basal Erk1/2 activity.

### 5.2.4. KDFs in serum free conditions have higher basal and IL-6 stimulated pAkt, which is absent in NDFs, but no effect of IL-6r blockade on basal pAkt levels is observed

Lastly, analysis of Akt stimulation was assessed (**Figure 5.6**). In 10% serum conditions, there was elevated basal pAkt levels in 2 out of 3 KDFs compared to NDFs and a small induction of pAkt after IL-6 stimulation across all cultures.

However, in serum free conditions, strikingly there was an absence of pAkt in NDFs basally and after IL-6 stimulation, whereas KDFs showed some basal pAkt and a marked increase after IL-6 stimulation (**Figure 5.6**). This IL-6 induced pAkt was highest in the same KDF that also had a strong induction of pStat3 in these conditions (**Figure 5.2**).

Tocilizumab treatment had no effect on pAkt, in NDFs and KDFs in 10% serum or serum free conditions (**Figure 5.7**).

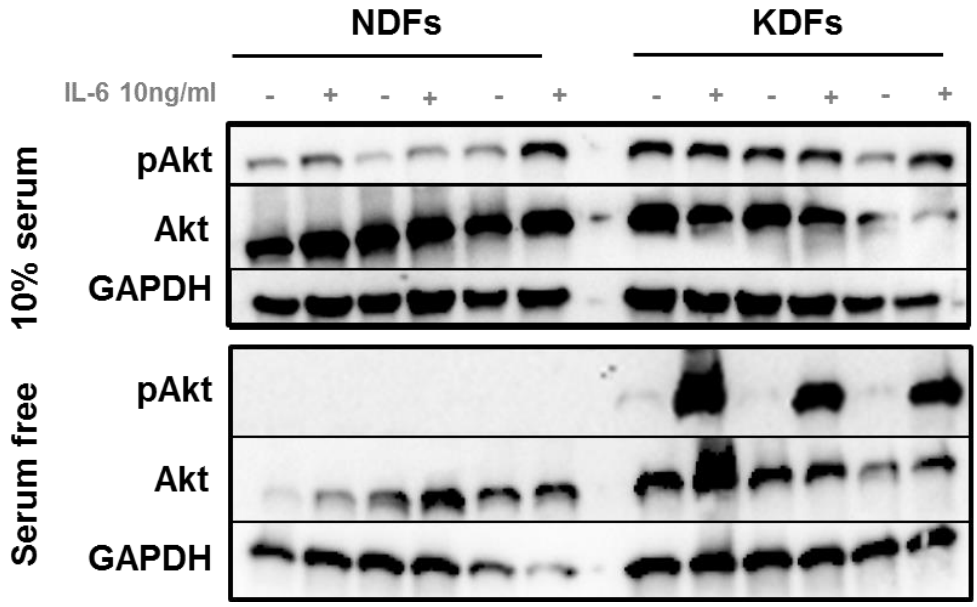



**Figure 5.6 - Western blot showing basal and IL-6 stimulated pAkt signalling in dermal fibroblasts**. n=3 NDF and KDF cultured in 10% serum and serum free conditions were lysed after a 30 minute stimulation with 10ng/ml IL-6. Blots were probed for pAkt, Akt and GAPDH to examine the level of Akt activation basally and after IL-6 stimulation.

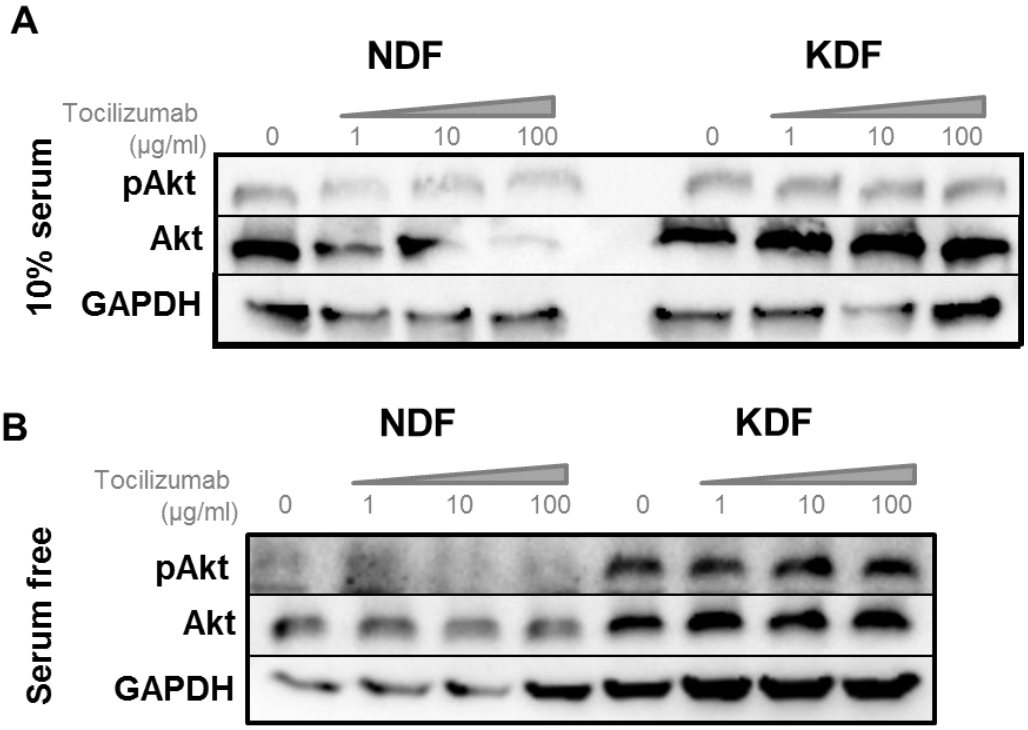



**Figure 5.7 - Consequence of IL-6r blockade on basal pAkt in NDFs and KDFs.** Western blot of NDF and KDF cultures in 10% serum (**A**) and serum free conditions (**B**) treated for 72 hours with increasing concentrations of the IL-6r blocking antibody Tociluzumab. Blots were probed for pAkt, Akt and GAPDH in order to identify the contribution of IL-6 in these cultures for driving basal Akt activity.

### 5.2.5. KDFs show elevated levels of IL-6r in serum free conditions compared to NDFs

Due to the dramatic differences seen in the signalling response to IL-6, especially in serum free conditions, the abundance of IL-6r was analysed in NDFs and KDFs. Cells were cultured for 24 hours prior to RNA extraction and RT-PCR. Primers published in (Vermes *et al.*, 2002) were used to distinguish both the full-length IL-6r (219bp) as well as the shorter length IL-6r (125bp) spliced isoform that gives rise to soluble IL-6r. Primers for ESP8 were also used as a reference gene. THP-1s were used as a positive control as they are known to express both forms of the receptor (Jones *et al.*, 1998). A 35 cycle PCR was conducted on 50ng of cDNA (further described above). PCR products were then run on a 2% agarose, Diamond-Dye containing gel alongside one well of Low Molecular Weight ladder to approximate product size. Gels were run for ~2 hours at 100V before visualisation.

In 10% serum conditions, it was apparent that NDFs and KDFs both expressed similar amounts of IL-6r transcript, with a low level of spliced isoform expression (**Figure 5.8A**). Band intensity was quantified and no difference between NDF and KDF IL-6r expression was identified (**Figure 5.8B**).

The PCR was then repeated in serum free conditions. In both cases, serum removal induced IL-6r expression (**Figure 5.8C**), however this was much more notable in KDFs which mirrored the increased signalling in response to IL-6 stimulation in serum free conditions (**Figure 5.8D**).

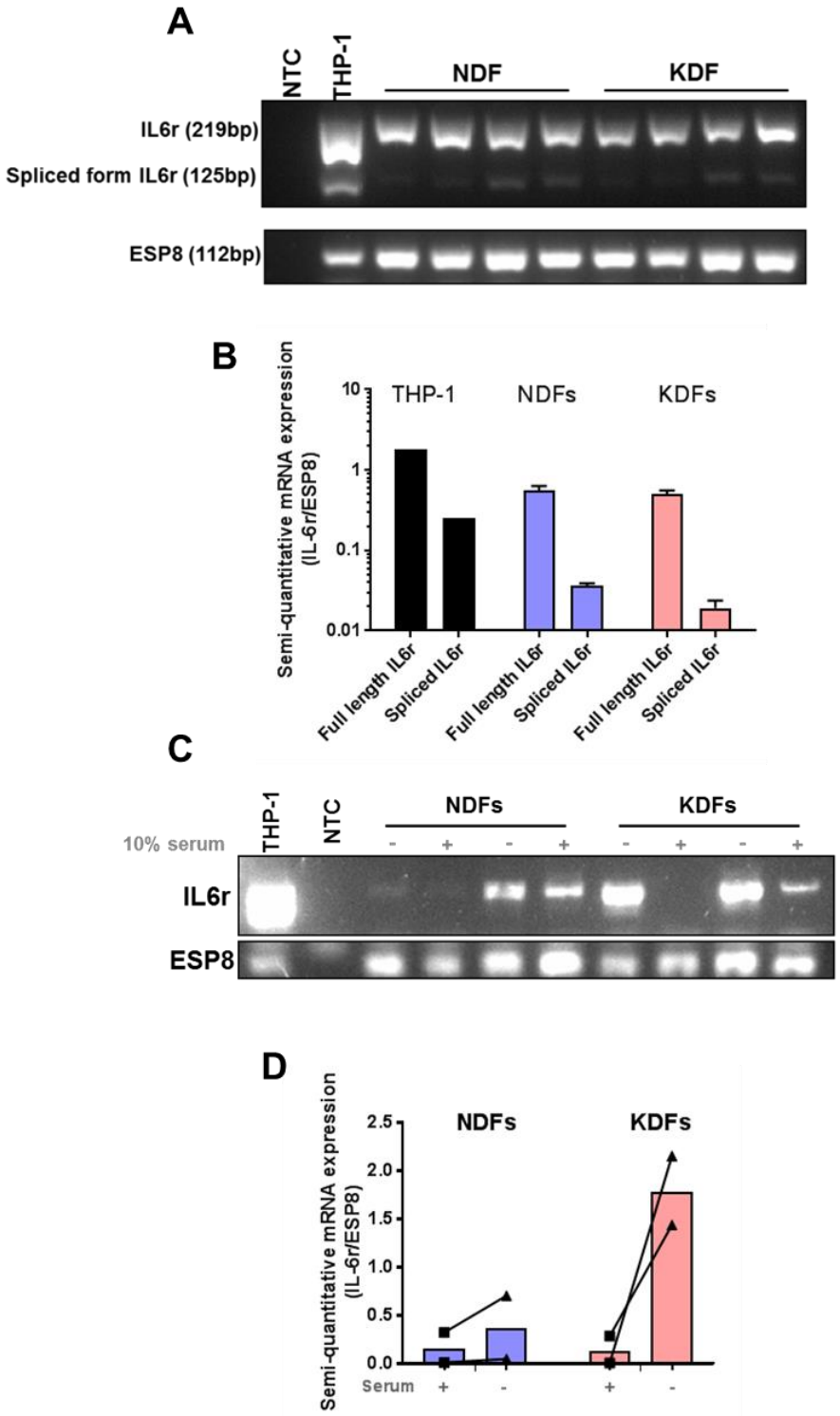



**Figure 5.8 - IL-6r mRNA expression in NDFs and KDFs.** (**A**) Full length IL-6r and the spliced isoform of IL-6r (which would generate the soluble form) were analysed by semi quantitative RT-PCR in n=4 NDFs and KDFs as well as THP-1 as a positive control. This result was then quantified (**B**) using ImageLab software, normalising band intensity of the sum of the two IL-6r bands to the intensity of the ESP8 band in n=4 NDFs and KDFs. (**C**) IL-6r was analysed by semi quantitative RT-PCR in n=2 NDFs and KDFs which were cultured in 10% serum or serum free conditions for 24 hours, as well as THP-1 as a positive control. Separation of full length IL-6r and the spliced isoform was not successful in this PCR. This was also quantified using ImageLab software (**D**).

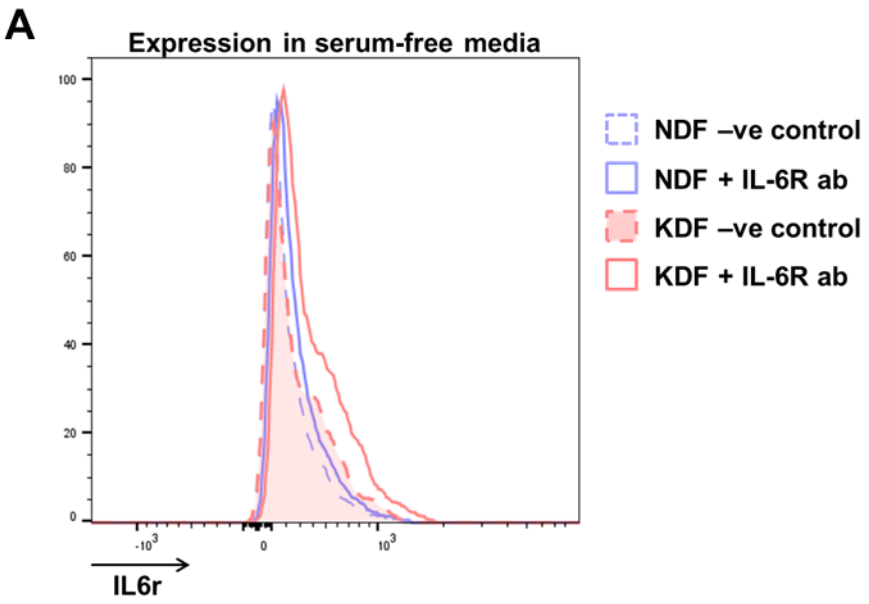
Flow cytometry was then used to examine IL-6r protein expression in an NDF and a KDF line cultured in 10% serum or serum free conditions. To capture both surface bound and internalised receptor, cells were split into two groups for extracellular and intracellular staining. It was postulated that due to the high level of IL-6r recycling (Zohlnhöfer *et al.*, 1992), intracellular staining would increase our ability to detect small differences in expression between NDFs and KDFs that may have biological significance. Intracellular staining was conducted by permeabilising cells with saponin to allow antibody internalisation. A REAfinity™ phycoerthyhin (PE) conjugated IL-6r antibody from Miltenyi Bio was used (clone REA291) and cells were also stained for viability using Fixable Viability Dye eFluor™ 506.

Compensation and sample acquisition were undertaken with assistance from Celine Trouillet using the FACSCanto (BD Biosciences). The resulting .fcs files were analysed using FlowJo, whereby events were gated on live singlet cells by forward/side scatter and Live/Dead dye negativity (above). During preliminary analyses, it became obvious that whilst KDFs appeared somewhat different by forward and side scatter then NDFs, appearing larger and more auto-fluorescent, the permeabilisation protocol also affected cell shape and fluorescence. Therefore, unstained and live-dead only controls were needed for each condition.

IL-6r expression was relatively low, with high levels of autofluorescence and no clear positive or negative population. However, when expression was represented as a histogram, there was a shift in fluorescence intensity, and therefore expression, in permeabilised, serum free KDFs, above the level of the fluorescent-minus negative control (cells from the same condition, stained only with live/dead dye) (**Figure 5.9A**). To quantify this further, geometric mean of the MFI was calculated in Flowjo for each acquired sample. These values were tabulated for both the fluorescent minus negative control and the fully stained sample in each condition (**Figure 5.9B**).

The level of expression above the fluorescent minus negative control was calculated by subtracting the geometric mean MFI of the negative control from the geometric mean MFI of the stained sample (**Figure 5.9C**). This showed that in non-permeablised cells only KDFs in serum free conditions showed detectable IL-6r expression. After saponin permeablisation the NDF and KDF lines in 10% serum and serum free conditions all had some detectable IL-6r. In these permeablised cells, serum removal approximately doubled the level of IL-6r expression in the KDF line tested, whilst having no clear effect in the NDF line.

An ELISA was also used to measure sIL-6r levels in the supernatant of fibroblasts cultures. Despite using concentrated supernatants which were collected after 72 hours of culturing, sIL-6r was undetectable in fibroblast cultures, suggesting sIL-6r levels in this system must be below 15.6pg/ml (data not shown). However, supernatants from the whole tissue *ex vivo* models were above this detection limit and did show increased production in keloid scar tissue compared to normal skin tissue (**Figure 5.10**).




B

| | | IL-6R (PE) MFI | | | |
|---|---|---|---|---|---|
| | | No permeablisation | | Saponin permeablisation | |
| | | -ve control | + IL-6r ab | -ve control | + Il-6r ab |
| 10% serum | NDF | 220 | 220 | 169 | 221 |
| | KDF | 297 | 297 | 235 | 290 |
| Serum free | NDF | 298 | 298 | 229 | 273 |
| | KDF | 340 | 363 | 286 | 385 |

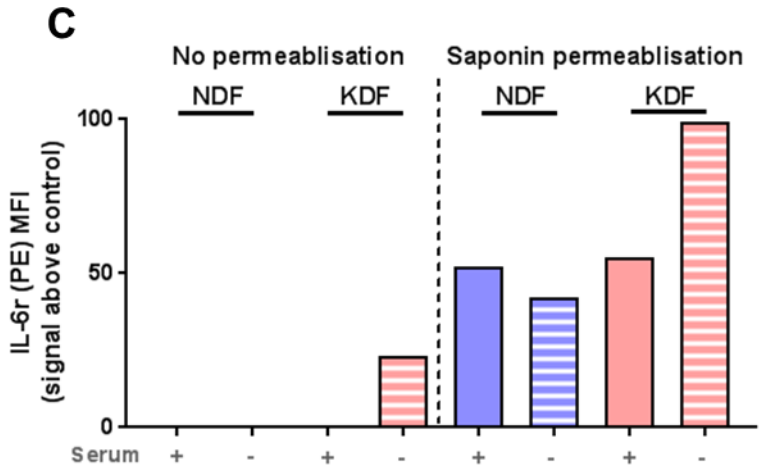



***Figure 5.9* - IL-6r expression in NDF and KDF.** (**A**) Flow cytometry analysis of IL-6r expression in NDF and KDF grown in serum free conditions where fluorescent minus controls are cells stained with live/dead dye only. Cells were permeabilised prior to staining, events were gated on live, singlet cells and expression was plotted as histograms to visualise expression shift. Mean fluorescence intensity (MFI) was tabulated as geometric mean for all samples including –ve controls (**B**). MFI was then plotted for NDF and KDF in 10% serum and serum free conditions in non-permeablised and permeabilised cells by subtracting the MFI value for the –ve control from the corresponding IL-6r stained sample (**C**).

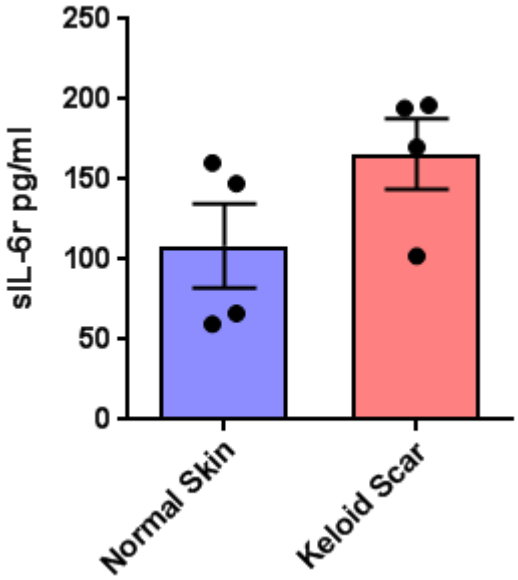



***Figure 5.10* - sIL-6r protein expression in tissue *ex vivo* models.** ELISA of 24 hour supernatants from n=4 normal skin and keloid tissue *ex vivo* cultures measuring sIL-6r levels relative to the standard curve, n=4, mean +/- SEM.

### 5.2.6. KDFs may have altered levels of GP130 and SOCS3 in serum free conditions compared to NDFs

qPCR was then used to investigate whether there may be a change in expression of other important IL-6 signalling machinery. mRNA levels of the main negative regulator of IL-6 signalling, SOCS3 (Croker *et al.*, 2003), and the co-receptor for IL-6 and other cytokine signalling, GP130 (Silver and Hunter, 2010), were examined. In 10% serum conditions, statistically significant differences in expression were not confirmed although there was an indication that there was slight increased GP130 and a trend for a decreased level of SOCS3 mRNA in a subset of KDFs compared to NDFs (**Figure 5.11A & B).**

After serum starvation, different responses were seen in NDFs vs KDFs. Upon serum removal, all 3 NDFs decreased gp130 expression, whilst 2 out of 3 KDFs increased GP130 expression (**Figure 5.11C**). The effect of serum removal on SOCS3 was less clear, however there appeared to be a more consistent decrease in expression in KDFs compared to NDFs (**Figure 5.11D**).

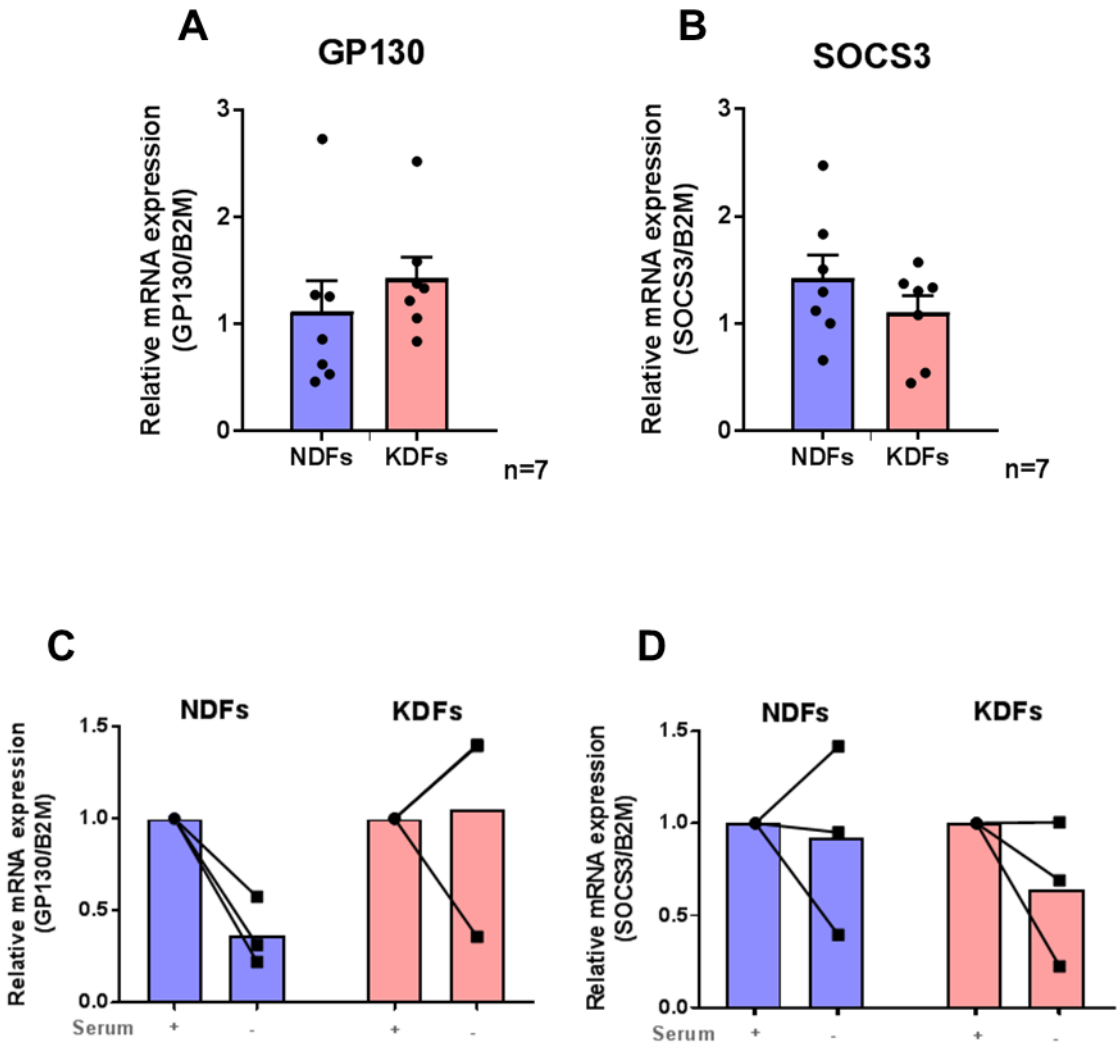



**Figure 5.11 - GP130 and SOCS3 mRNA levels in NDFs and KDFs.** GP130 (**A**), the co-receptor needed for IL-6 signalling , and SOCS3 (**B**), a key negative regulator of IL-6 signalling, were analysed by qPCR and relative expression was calculated using a standard curve and normalised to the reference gene B2M, n=7, mean +/- SEM. GP130 (**C**) and SOCS3 (**D**) were analysed by qPCR in n=3 NDFs and KDFs which were cultured in 10% serum or serum free conditions for 24 hours, relative expression was calculated using a standard curve and normalised to the reference gene B2M.

### 5.2.7. I-BET151 can reduce IL-6 secretion by NDFs and KDFs

Since BET inhibition had previously been reported to be anti-inflammatory (Belkina, Nikolajczyk and Denis, 2013), and BET proteins have been documented to interact with NF-κB signalling (Belkina, Nikolajczyk and Denis, 2013) and specifically the IL-6 promoter region via CBP (Barrett *et al.*, 2014), the effect of BET inhibition on fibroblast IL-6 production was examined.

NDFs and KDFs were treated with 1μM I-BET151 or 0.01% DMSO (vehicle control), for 24 hours before analysing the level of IL-6 in the supernatants by ELISA. This analysis showed a statistically significant decrease in IL-6 in both NDF and KDF supernatants, with a particularly striking decrease seen in KDFs that had the most elevated IL-6 levels in the paired controls (**Figure 5.12A)**.

To briefly examine how the temporal dynamics of this inhibition, supernatants were taken from I-BET151 and vehicle treated wells of a single NDF line over a number of timepoints: 1, 2, 4, 8, 24 and 72 hours (**Figure 5.12B)**. The I-BET151 mediated decrease in IL-6 secretion was not apparent until 24 hours.

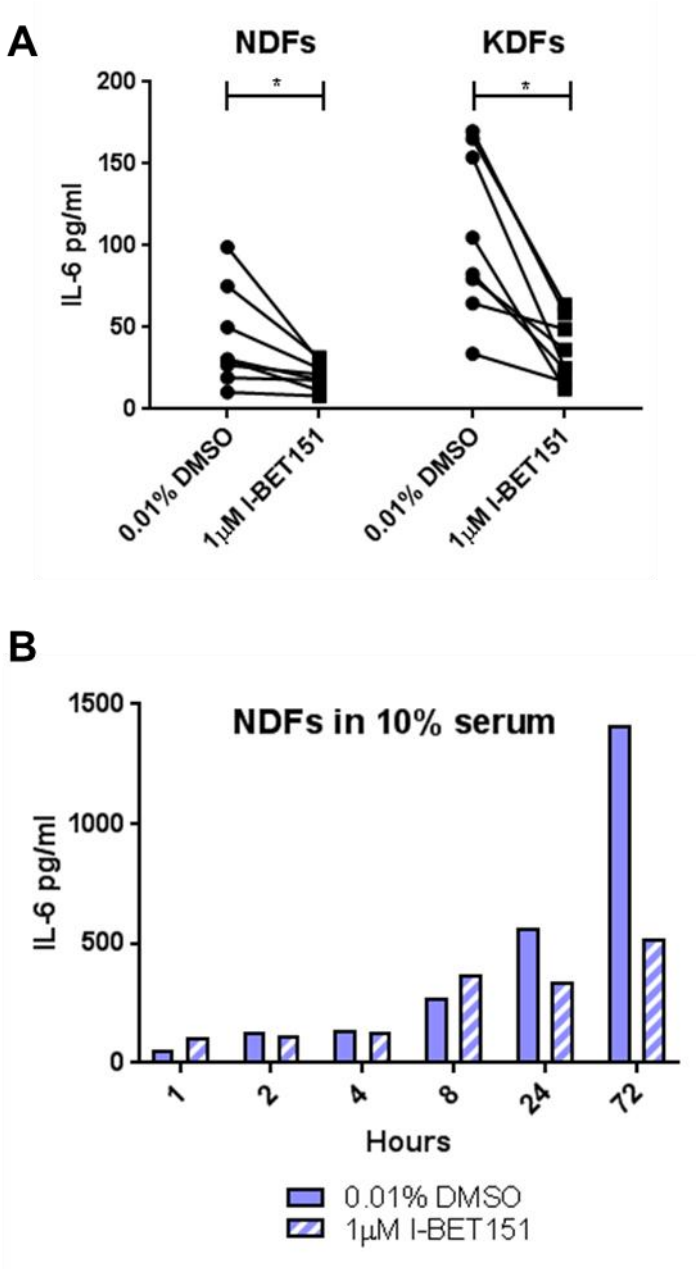



***Figure 5.12*** **- Effect of BET inhibition on IL-6 secretion in NDFs and KDFs**. IL-6 ELISA of (**A**) 24 hour supernatants from n=8 NDF and KDF cultures in 10% serum, treated with 0.01% DMSO or 1μM I-BET151, *, $p<0.05$ Mann-Whitney test. (**B**) Time course of NDF in 10% serum, treated with 0.01% DMSO or 1μM I-BET151 showing IL-6 production over a 72 hour period.

### 5.2.8. I-BET151 can reduce IL-6 induced signalling in KDFs in a transcription-dependent manner

Given the strong effect that I-BET151 appeared to have on IL-6 production, we next explored the effect of I-BET151 on downstream signalling. Cells were pre-treated with 1μM I-BET151 for 2 hours before 30 minute 10ng/ml IL-6 stimulation as represented in the schematic (**Figure 5.13**).

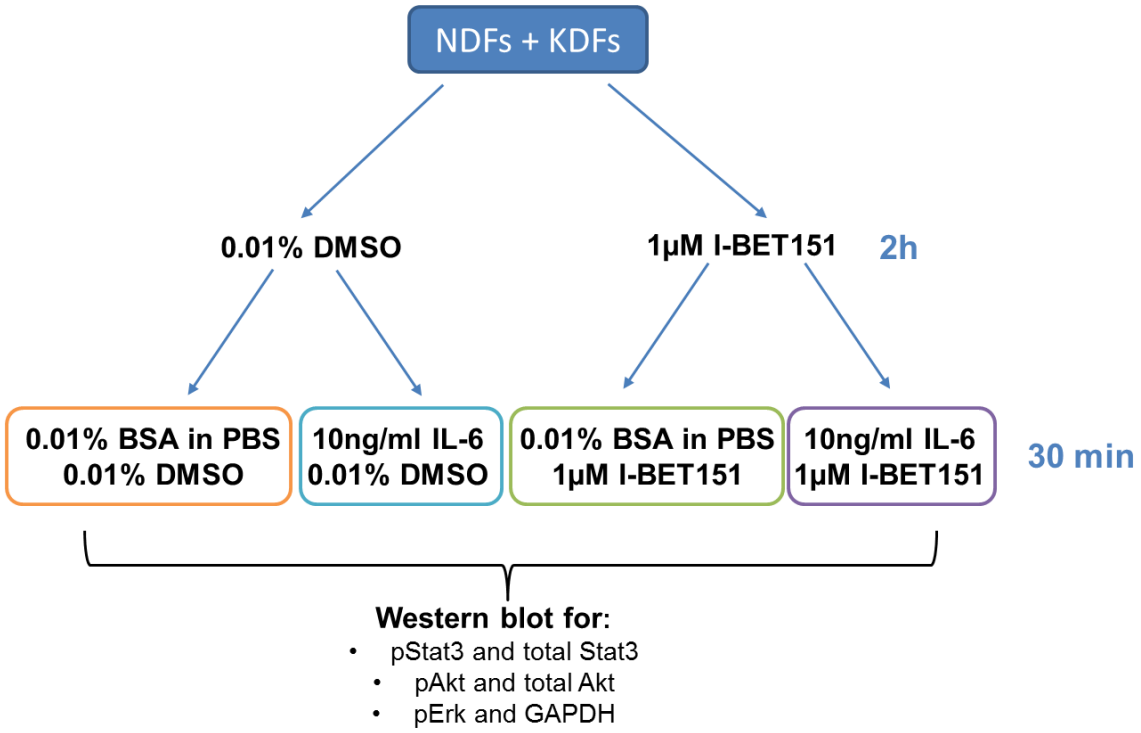



***Figure 5.13* - Schematic representing experimental setup to investigate BET inhibition effect on signalling.** Diagram showing treatment regime for experiment to investigate the effect of BET inhibition on basal and IL-6 signalling through Stat3, Erk1/2 and Akt, cells were pre-treated for 2 hours with I-BET151 or vehicle control, followed by 30 minute stimulation with IL-6 or vehicle control, still in the presence of absence of I-BET151. Protein was then lysed and analysed by western blot for the three signalling pathways.

A complete experiment with all four conditions in one NDF and one KDF line was run on a single western blot to directly compare the basal and IL-6 induced signalling activity in the two cell types. This experiment was repeated in both 10% serum and serum free conditions in n=3 NDF and KDF lines. A representative blot is shown (**Figure 5.14**) and quantification was conducted for the pStat3 and pAkt response, normalised to GAPDH, in all three biological NDF and KDF replicates (**Figure 5.15**). Quantification was not possible for pErk due to blot quality and normalisation to GAPDH rather than the total form of each signalling molecule was chosen due to possible inefficient stripping before reprobing of blots.

When examining IL-6 induced signalling pathways that were decreased after I-BET151 in 10% serum conditions, two KDFs showed decreased levels of two signalling pathways whilst only one NDF showed slight decreased levels of just one signalling pathway (Stat3) which is the blot shown (**Figure 5.14**). In 10% serum conditions there was a variable response to I-BET151 (**Figure 5.15**). However, responses were variable and in some cases (one NDF and one KDF line), I-BET151 appeared to cause slight increased levels of IL-6 induced signalling (**Figure 5.15**)

In serum free conditions, the ability of I-BET151 to inhibit IL-6 signalling was much more striking in KDFs, with all three KDF showing decreases in multiple signalling pathways (**Figure 5.14**, **Figure 5.15**), whereas only one NDF showed any I-BET151 mediated decrease in IL-6 induced signalling, with two pathways decreased.

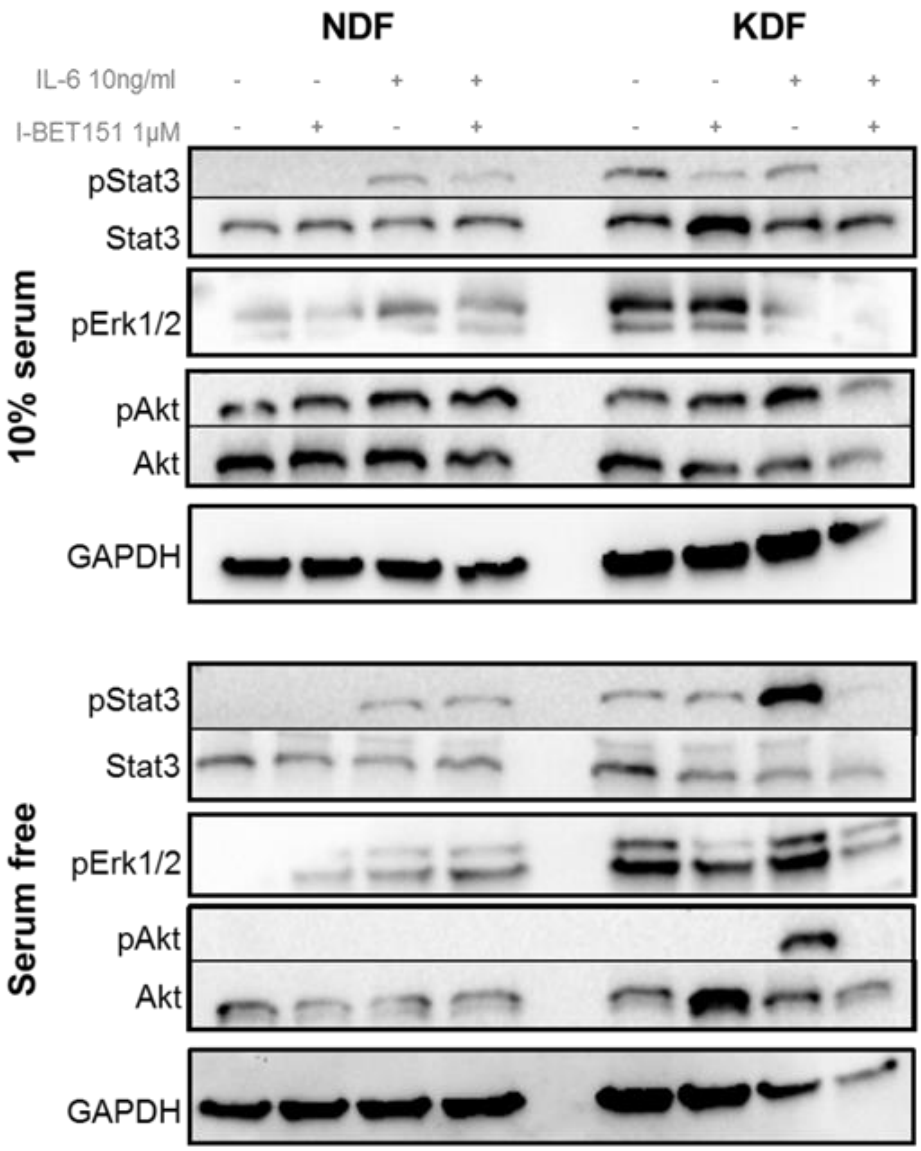



***Figure 5.14*** **- Effect of BET inhibition on basal and IL-6 induced signalling.** Representative western blot of NDF and KDF cultures treated with 1μM I-BET151 or 0.01% DMSO vehicle (2 hour pre-treatment) before IL-6 treatment for 30 minutes. Blots were probed to examine the effect of BET inhibition on Stat3, Erk1/2 and Akt activation basally and after IL-6 stimulation.

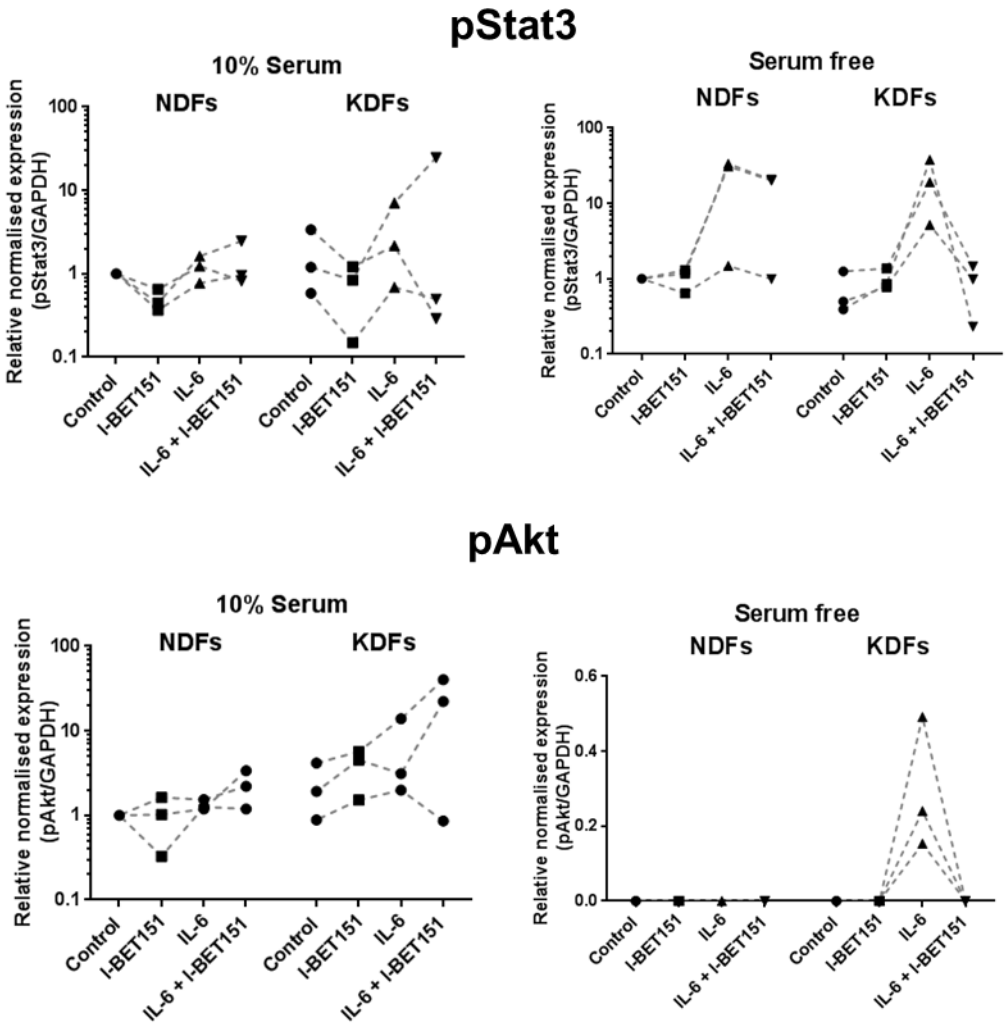



***Figure 5.15*** **- Summary of effect of BET inhibition on basal and IL-6 induced signalling**. Summary of the effect of 1μM I-BET151 on IL-6 induced Stat3 and Akt activation in 10% serum and serum free conditions in n=3 NDFs and KDFs.

As BET has known interactions with histone and non-histone proteins (Xu and Vakoc, 2014; Xiao *et al.*, 2015), it was unclear whether the I-BET151 mediated decrease in signalling was dependent on transcriptional changes. To explore this, Actinomycin D was used as a transcriptional inhibitor. Actinomycin D is a bacterial compound that intercalates into DNA and prevents the progression of RNA polymerase (Koba and Konopa, 2005). Historically it has been used as a chemotherapy drug, however it has been used in laboratory experiments as a transcriptional inhibitor since the 1970s (Koba and Konopa, 2005).

To determine an appropriate dose of Actinomycin D in our model, an NDF line was treated with increasing concentrations of Actinomycin D based on previous literature for 2.5 hours. RNA was isolated, cDNA generated and qPCR was conducted. SOCS3 was chosen as a readout of inhibition because literature suggested rapid degradation of this transcript within the time span of this experiment (Siewert *et al.*, 1999). B2M was also analysed as a reference gene. This pilot analysis suggested that the two highest concentrations were the most effective at reducing transcription (**Figure 5.16A**).

Then, a KDF line in 10% serum conditions was treated as **Figure 5.13**, but in the absence or presence of 1µg/ml or 2µg/ml Actinomycin D throughout the experiment (added at the same time as I-BET151). Again, protein was lysed and analysed by Western blot. It was found in the absence of Actinomycin D as expected there was an increase in pStat3 upon IL-6 stimulation and a decrease when treated with I-BET151 (**Figure 5.16B**). However, in the presence of Actinomycin D, the effect of I-BET151 was lost. Also, there was some indication that in the presence of 1µg/ml Actinomycin D the resting level of pStat3 was decreased and the induction of pStat3 after IL-6 treatment was slightly increased, which could be linked to a decrease in SOCS3 as we know this is rapidly degraded transcript (**Figure 5.16A**).

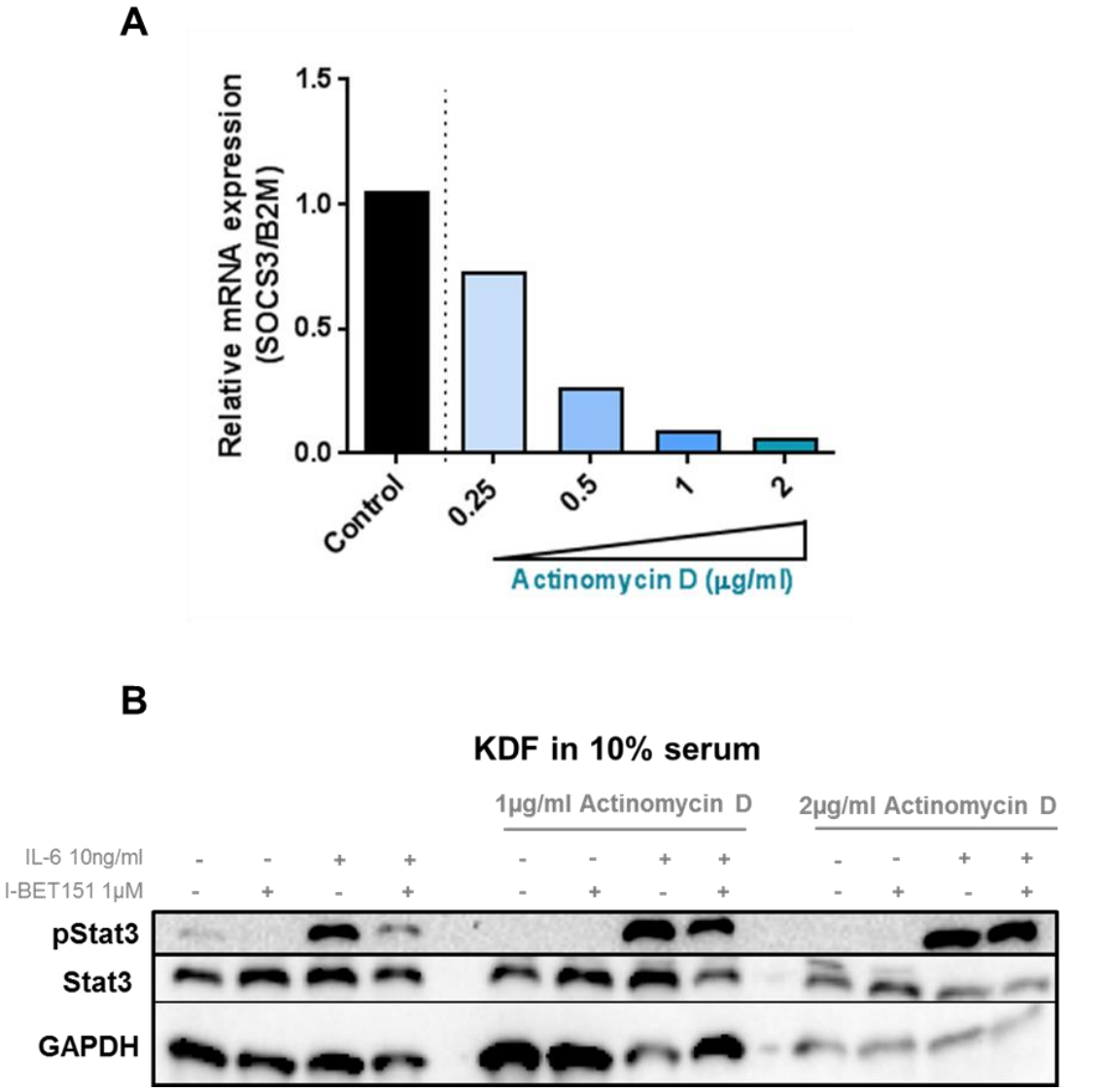



***Figure 5.16*** **- Effect of transcription inhibition on I-BET151 mediated signalling decrease.** (**A**) qPCR was conducted on NDFs treated with increasing doses of the transcriptional inhibitor Actinomycin D for 2.5 hours to find an appropriate dose to prevent transcription in these cells. SOCS3 was chosen due to its short stability (~15 minutes). Relative expression was calculated using a standard curve and normalised to the reference gene B2M**.** (**B**) Western blot of KDF treated as described in Figure 1-13, +/- treatment with 1µM I-BET151 or 0.01% DMSO vehicle (2-hour pre-treatment) +/- 10ng/ml IL-6 stimulation, but in the absence and presence of the transcriptional inhibitor Actinomycin D (added at the same time as I-BET151) to examine whether the I-BET151 mediated decrease in signalling was reliant on transcription. Blots were probed to examine Stat3 activation.

### 5.2.9. I-BET151 causes a small reduction in IL-6r expression

Having determined that the I-BET151 mediated reduction of signalling was likely due to a transcriptional change, we examined the effect of I-BET151 on IL-6r expression which we saw as a potential regulation point for signalling. RT-PCR was used to measure IL6r transcript following the 2.5-hour experiment depicted in **Figure 5.13** in serum free conditions in a NDF and KDF line.

As found previously (**Figure 5.8C**), the KDF showed higher IL-6r mRNA abundance than the NDF in serum free conditions. The 30 minute IL-6 stimulation did not affect expression of IL6r, however I-BET151 treatment did appear to reduce expression slightly in both the NDF and KDF (**Figure 5.17**). However, this reduction was relatively mild in comparison to the marked reduction in signalling and was similar in the NDF and KDF, suggesting that this is not a KDF specific phenomenon.

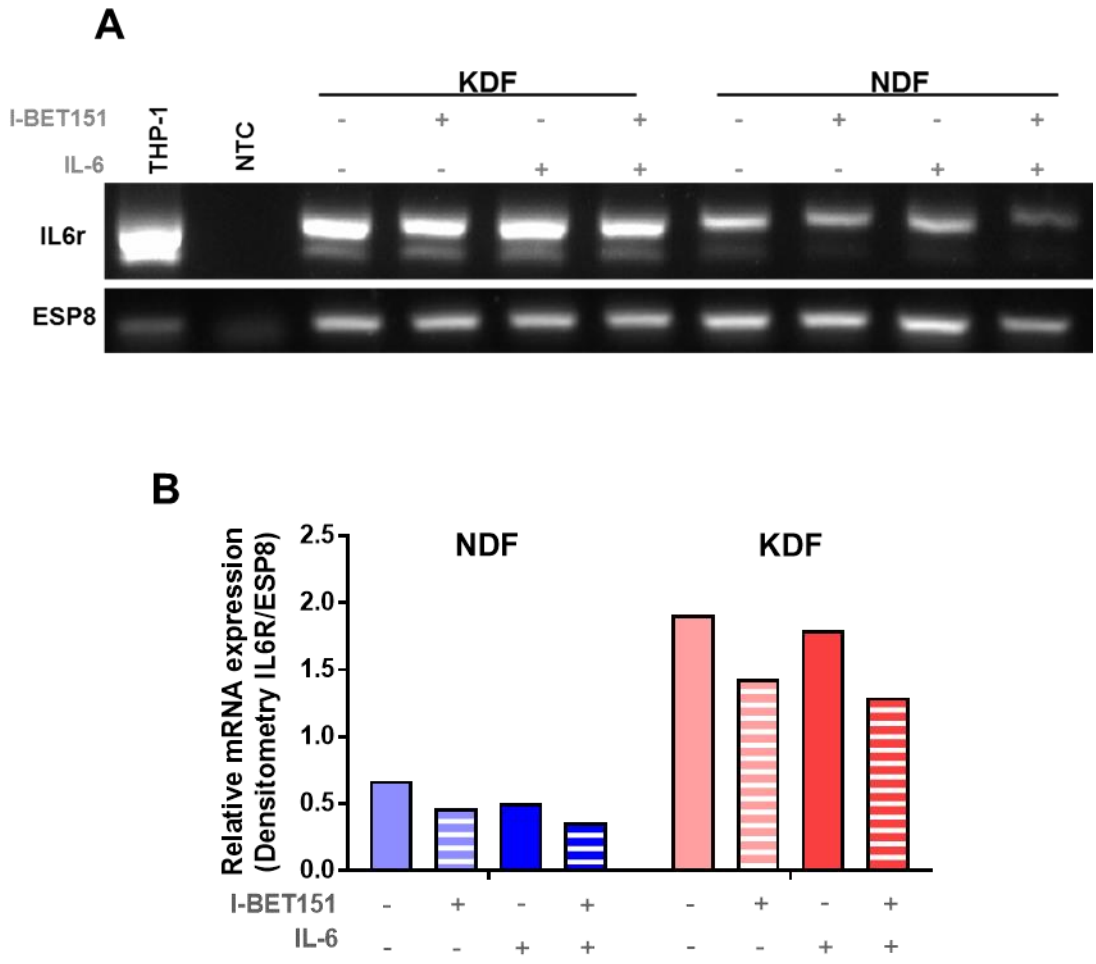



***Figure 5.17*** **- IL-6r expression after IL-6 stimulation and BET inhibition.** Full length IL-6r and the spliced isoform of IL-6r (which would generate the soluble form) was analysed by semi quantitative RT-PCR in a NDF and KDF line in four conditions, treated as described in Figure 1-13, +/- treatment with 1μM I-BET151 or 0.01% DMSO vehicle ( 2 hour pre-treatment) +/- 10ng/ml IL-6 stimulation as well as THP-1 as a positive control (**A**). This result was then quantified (**B**) using ImageLab software, normalising band intensity of the sum of the two IL-6r bands to the intensity of the ESP8 band.

## 5.3. Discussion

### 5.3.1. IL-6 ligand production

The increase in IL-6 production seen in both keloid tissue *ex vivo* cultures and in fibroblast monolayer cultures confirms what has previously been reported in multiple studies (Xue, McCauley and Zhang, 2000; Ghazizadeh *et al.*, 2007; Zhang *et al.*, 2009). Interestingly, there appears to be some stratification of patients with some IL-6 over-producers and some within the range of normal tissue. This was also mirrored in other Luminex data from the lab. Heightened IL-6 has been shown to be an indicator of poorer prognosis in cancer (Egler *et al.*, 2008; Khan *et al.*, 2012; Chen *et al.*, 2013) and in systemic sclerosis (Khan *et al.*, 2012), and therefore could be used as a factor to stratify patients. It would be valuable to better characterise the tissue from each keloid patient and correlate this to IL-6 production, for example by investigating whether IL-6 overproducing cells derived from keloids that are actively growing or particularly inflamed, to better understand what may drive, or be a consequence of, high IL-6 in keloids.

Fibroblast cultures up to passage 7 were used in the analysis of IL-6 production suggesting that the ability of KDFs to produce increased levels of IL-6 is retained in their cellular 'memory'. This may be due to priming *in vivo* by the keloid environment, including contact with other cell types. The current literature educates us on the strong memory of fibroblasts related to their anatomical origin (Ivanov *et al.*, 2016) and previous mechano-stress (Yang *et al.*, 2014), thought to be driven at least in part by exposure to environmental cues that cause epigenetic changes. Therefore, it is likely that the chronic inflammatory environment of a keloid scar also imparts some level of epigenetic 'memory' onto KDFs that results in behavioural changes that are retained *in vitro*. A pro-inflammatory memory has been described in synovial fibroblasts, from both healthy and diseased (RA) joints, whereby repeated proinflammatory cytokine stimulation (TNFα or IL-1α) caused an increased inflammatory response with higher levels of IL-6 production (Crowley *et al.*, 2017).

Whilst KDFs produced around double the amount of IL-6 compared to NDF, keloid tissue produced around 10 times the amount of IL-6 compared to normal skin. The high level of IL-6 produced in keloid tissue *ex vivo*s may be reflected by the increased cellularity and/or increased depth of tissue biopsy, however it may also suggest that there are other cells present within the keloid tissue that are responsible for IL-6 production such as inflammatory macrophages (Bagabir *et al.*, 2012a). The definite identity of IL-6 producing cells in keloids is difficult to ascertain given that we do not yet fully understand the cellular composition of these scars.

However, it does show that there are high levels of IL-6 in the keloid environment, which we have demonstrated that fibroblasts are capable of sensing.

IL-6 production by the single NDF and KDF line in serum free conditions was much reduced (around 10 to 50 fold less) compared to 10% serum conditions, however it was still clear that the KDF culture could produce around double the amount of IL-6 compared to the NDF culture. Cellular cytokine production in serum free condition is much less characterised than in standard culturing conditions, especially in the keloid field. One studied found that in serum free conditions NDFs produced almost no TGF-β1 whereas KDFs produce moderate levels over a 72 hour period (Hanasono *et al.*, 2003). As TGF-β1 is known to drive IL-6 production in fibroblasts (Eickelberg *et al.*, 1999), this may explain some of the disparity seen in IL-6 production between NDF and KDF in serum free conditions. Serum may also stimulate fibroblasts in culture to become more inflammatory, and therefore produce more IL-6, as a part of a wound response to serum that has been previously described (Iyer *et al.*, 1999). The possible activation of downstream signalling in both NDFs and KDFs was examined, at the basal level and after IL-6 stimulation, for three main signalling pathways: Stat3, Erk and Akt (Figure 1.6).

### 5.3.2. Basal and IL-6 induced signalling

Serum also appeared to activate fibroblasts, inducing higher basal pStat3 and pAkt in both NDFs and KDFs, although had little effect on basal pErk levels. The content of serum is somewhat uncharacterised, but is known to include a range of growth factors that may be capable of inducing both Stat3 and Akt signalling (Adi *et al*. 2001; Zhang *et al*. 2005). Many suggest that serum is very unnatural, cells are not bathed in serum *in vivo*, therefore the removal of serum enables us to look at a more simplified picture of signalling in the absence of any exogenous growth factors that may be present in serum. The removal of serum in our cultures has unravelled marked signalling differences that were not seen in 10% serum conditions. Additionally, the presence of 10% serum in culture dampened the effect of IL-6 stimulation on cell signalling, with only a small induction of pStat3, inconsistent induction of pAkt and no induction of pErk across the fibroblast cultures tested. The mean IL-6 production in the keloid *ex vivo* cultures was 4.6ng/ml, suggesting that the concentration of human recombinant IL-6 used in stimulation experiments (10ng/ml) is appropriate. Upon serum removal, basal pErk was not affected in either cell type. Disparity in the serum starvation response has been observed in a study focussing on cancer that saw upregulation of phosphorylated protein and total protein (including Akt, Erk and Stat3 pathway proteins) in a range of glioma cell lines but not adenocarcinoma cell lines after serum starvation (Levin *et al.*, 2010). The authors postulate that

this response is a molecular adaption to stress as the glioma cells also show resistance to apoptosis (Levin *et al.*, 2010), which may be mirrored in keloids as resistance to apoptosis has also been reported.

It is possible that IL-6 contributes to KDF basal pStat3 in serum free conditions as the IL-6r blocking antibody Tocilizumab reduced pStat3 levels in these cultures. However, there was no clear effect of Tocilizumab on pAkt levels, suggesting there is another intrinsic factor being released by fibroblasts, in the absence of serum, that is driving basal Akt signalling. There are a variety of other candidates that may be able to drive signalling including a range of cytokines (IL-9, IL-10, IL-11, IL-12, IL-17A, IL-27, TNFα, OSM, LIF, MCP-1) and growth factors (GCSF, GMCSF, HGF, EGF, FGF or PDGF). However, no IL-17A or IL-10 was detected in our fibroblast cultures by ELISA (data not shown), making their involvement unlikely. Similar experiments to the Tocilizumab investigation, using the tyrosine kinase inhibitor Imatinib showed some decrease in basal pAkt in both 10% serum and serum free conditions (data not shown), suggesting that PDGF-BB may be responsible for some of this signalling, although there was no effect on pStat3.

In serum free conditions, NDFs had no detectable levels of pAkt or pStat3, whilst KDFs still showed low levels. Additionally, KDFs tended to induce higher levels of pStat3 than NDFs after IL-6 stimulation in serum free conditions. KDFs also responded to IL-6 stimulation in serum free conditions with an increase in pErk and a striking increase in pAkt that was completely absent in NDFs. It is possible that there is a component within serum that is blocking signalling triggered by IL-6 stimulation, however it is also possible that a component within serum drives basal signalling prior to IL-6 stimulation, which would in turn promote constitutive expression of a negative regulator of signalling. In this project, we have observed a possible decrease in SOCS3 expression after serum removal, although this was not consistent and was in limited replicates. The finding that NDFs and KDFs have disparity in their intrinsic signalling response to an external inflammatory stimuli may present important implications for keloid surgery, as ‘wounding’ of a keloid scar may not elicit the same inflammatory response seen in normal skin.

### 5.3.3. Stat3 signalling

All three signalling pathways were activated, to some extent, in KDFs in serum free culture in a way that wasn’t seen in NDFs. This indicates that these pathways could have some importance in keloid fibroblast behaviour and pathology. Stat3 is the most well characterised signalling molecule downstream of IL-6 and also the most studied in its role in keloid pathology. pStat3

has been reported to be upregulated in severe scars but appears to be inconsistent in some experiments, which in some cases may be due to serum in culture media as we have observed. pStat3 has been shown to be elevated in hypertrophic scar IHC however the interpretation of this data is difficult given the damage to tissue sections seen in this paper (Ray *et al*., 2013). More convincing evidence that pStat3 is elevated in keloid scars has been shown by IHC and western blot (Lim *et al.*, 2006). This study also showed increased total Stat3 which may indicate a higher capability of these cells to induce Stat3 signalling after stimulation (Lim *et al.*, 2006) although this is not something we observed in our western blot experiments. In a later study, the Lim group showed that in low serum conditions (0.5% serum) IL-6 as well as OSM caused induction of pStat3, although they saw no difference between NDFs and KDFs (Lim *et al.*, 2009). Increased pStat3 has been observed in a hypertrophic scar fibroblast line (HSDF) compared to an NDF line, and the authors also state that there was an increase in pStat3 in the HSDF after a co-stimulation with IL-6 and sIL-6r but the data for this experiment is not shown (Ray *et al.*, 2013).

Interestingly, a more complex co-culture system with patient-matched fibroblasts and keratinocytes has shown a decrease in pStat3 which indicates some level of negative regulation in this cell system, although they do not see the baseline differences between NDF and KDFs that we have observed (Lim *et al.*, 2009). Additionally, although this is a more complex model, it may not be much more relevant to the *in vivo* scenario, given the other cells resident in keloids that may be contributing towards the production of IL-6 (and other activating analytes including sIL-6r) such as inflammatory macrophages, which could further drive Stat3 signalling.

Mechanistically, pStat3 is thought to be very important in a fibrotic context (Knight, Mutsaers and Prêle, 2011) and could indeed be relevant in keloid scars. It has been shown that Stat3 activation can lead indirectly to Smad3/TGF-β signalling through the induction of the transcription factor Gremlin in systemic sclerosis skin fibroblasts (O'Reilly *et al.*, 2014). Given the reports of overexpression of TGF-β1 in keloids (Jagadeesan and Bayat, 2007), this could be a shared mechanism in the two diseases. A small number of studies have demonstrated inhibition of Stat3 signalling that may be clinically beneficial, with a decrease in a range of ECM proteins, proliferation and migratory behaviour after treatment with Stat inhibitor or Stat3 siRNA (Lim *et al*., 2006; Ray *et al*., 2013). One paper suggests the reduction in phenotype is to the level of the typical NDF (Lim *et al.*, 2006), although no clear data have been shown to support this.

In our investigations we used a Stat3 antibody against the phosphorylated single tyrosine residue (Tyr705) which is thought to be the main point of regulation of Stat3 activity, as this allows dimerization and consequent downstream activation (Schindler and Darnell, 1995). It has

been shown that maximal Stat3 activation can be caused by a secondary phosphorylation at a single serine residue (Ser727) (Schuringa *et al.*, 2000). There is some evidence that pStat3 Ser727 is also increased in keloids (Lim *et al.*, 2006) and it would be insightful to investigate this in our cultures as this may explain the heightened response to IL-6 stimulation seen in KDFs in serum free conditions.

### 5.3.4. Erk signalling

When Erk signalling was examined after IL-6 stimulation, only KDFs in serum free conditions appeared to be able to increase pErk levels, with KDFs in 10% serum and NDFs in all conditions having no pErk response. Interestingly, it has been suggested that Erk signalling can function to inhibit Stat3 signalling (Sengupta *et al.*, 1998), which may be an antagonistic 'damage control' mechanism that kicks in KDFs which are highly phosphorylating Stat3. Supporting this theory, the KDF in serum free conditions that had the highest induction of pStat3 after IL-6 stimulation also had the highest induction of pErk (1$^{st}$ KDF). Intriguingly, the inhibitory action of Erk signalling is thought to be caused by post-translational modification of IL-6 signalling machinery (Sengupta *et al.*, 1998), although the identify of these is not described.

pErk, as well as pAkt has been shown to be increased in NDFs and KDFs that are co-cultured with keratinocytes and are thought to be important for proliferation (Phan *et al.*, 2003) and for the production and modification of ECM proteins (Lim *et al.*, 2003). Pharmacological inhibition of both Erk and Akt pathways in these systems showed decreased levels of collagens, laminin and fibronectin and changed molecular mass in the case of fibronectin (Lim *et al.*, 2003). Mechanistically, Erk signalling has also been linked to the nuclear translocation of NF-κB in prostate cancer (Rodriguez-Berriguete *et al.*, 2010), which could further propagate inflammatory transcription that has been documented in keloids (Messadi *et al.*, 2004). Erk is also thought to mediate the exaggerated transcriptional responses by KDFs compared to NDFs when under mechanical strain, as the upregulation of TGF-β1 and collagen Iα genes were inhibited with an Erk inhibitor U0126 (Wang *et al*. 2006).

### 5.3.5. Akt signalling

The most striking difference in signalling after serum starvation was the dramatic induction of Akt signalling in KDFs that is completely absent in NDFs. This signalling could be interpreted

as a stress response that is lacking in NDFs. Akt signalling is often described as pro-proliferative or a pro-survival mechanism and is often constitutively active in cancer (Nicholson and Anderson, 2002). It is clear from the literature that as well as a plethora of possible downstream substrates of Akt there are many interacting proteins that can modulate its activity including its impact on other signalling pathways (**Table 5.1**) (Brazil, Park and Hemmings, 2002). Hence the literature in this area is highly conflicting and makes it extremely difficult to postulate what biological effect Akt may be mediating in a keloid context although it has been linked to proliferation (Wegiel *et al.*, 2008), survival (Horowitz *et al.*, 2004), metabolism (Robey and Hay, 2009), and migration (Franke *et al.*, 2003). Similarly to Stat3, we only examined phosphorylation of a single residue (Ser473), however phosphorylation at Thr308 also appears to be important to fully activate Akt activity (Gao, Moten and Lin, 2014) so again it would be useful to probe our blots with an antibody against this residue also.

The relevance of Akt signalling in collaboration with Stat3 signalling is unclear. It has been proposed that in scar fibroblasts, IL-10 stimulation can stimulate cross talk between Stat3 and Akt which drives an anti-fibrotic response (Shi *et al.*, 2014). However, another paper shows that co-operation of Stat3 and Akt signalling can lead to faster tumour progression in a mouse model of prostate cancer (Blando *et al.*, 2011).

Work in our laboratory by MSc student Yvonne Abrokwah suggests that despite differences in IL-6 induced signalling in pathways that may be classically associated with proliferation, there is no difference in the proliferative response between NDF and KDFs treated with IL-6 and/or Akt inhibitor for 72 hours (data not shown). However, Akt signalling is also known for its role in apoptosis and with clues in the literature that KDFs may be more resistant than NDFs to apoptosis (Ladin *et al*. 1998; Sayah *et al*. 1999; Ishihara *et al*. 2000). Resistance to apoptosis is a relevant clinical issue and may be due to mechanical load as modelling scar contractures in mouse wounds similar to human hypertrophic scars does induce pro-survival Akt signalling (Aarabi *et al.*, 2007). It has been shown in lung fibroblasts that TGF-β1 induced resistance to apoptosis appears to be mediated through Akt signalling and the release of a soluble factor, as conditioned media from TGF-β1 treated cells can activate Akt signalling in unstimulated cells (Horowitz *et al.*, 2004). It would be interesting to conduct a similar experiment, transferring conditioned media from serum free, IL-6 stimulated KDFs to NDFs and comparing the Akt signalling response compared to a direct serum free IL-6 stimulation. This would enable us to decipher whether it is a secreted factor that may be mediating this aberrant response.

***Table 5.1* – Known substrate and interacting proteins of Akt (PKB), taken from Brazil *et al*, 2002.**

Table 1. Examples of PKB Interacting Proteins

| Interacting Protein | Biological Effect | Endogenous Interaction Demonstrated? | Ref |
|---|---|---|---|
| Nonsubstrates | | | |
| TCL1 | Increases PKB kinase activity and nuclear translocation | + | 1–4 |
| CTMP | Inhibits S473 phosphorylation on PKB | + | 5 |
| Hsp90/Cdc37 | Protection from dephosphorylation, degradation of PKB, and recruiting its substrates | + | 6–8 |
| Hsp27 | Activation of kinase activity | − | 9 |
| GLUT4 vesicles | Exact role unclear, important for insulin-stimulated glucose uptake | + | 10, 11 |
| JIP1 | Negative regulation of the JNK pathway | − | 12, 13 |
| Axin-GSK3β-Dvl | Mediates GSK3α/β regulation in Wnt signaling pathways | + | 14 |
| PKC α,δ, ζ | (Functional relevance remains to be determined) | − | 15–17 |
| Keratin 10 | Inhibits intracellular translocation of PKB | + | 18, 19 |
| Grb 10 | Translocation of PKB to the plasma membrane | + | 20 |
| Myosin II | (Functional relevance remains to be determined) | − | 21 |
| IMPDH | Regulation of GTP biosynthesis | − | 22 |
| APPL | (Functional relevance remains to be determined) | + | 23 |
| G protein βγ subunit | (Functional relevance remains to be determined) | − | 15 |
| Btk | Positively activates PKB in $H_2O_2$ signaling in B cells | + | 24 |
| Substrates | | | |
| Mdm2/Hdm2 | Nuclear translocation of Mdm2, decreases p53 levels | + | 25–28 |
| p21Cip/WAF1 | Enhances protein stability of p21 and promotes cell survival | + | 29 |
| EDG-1 | Activation of GPCRs in G(i)-independent manner | + | 30 |
| BAD | Phosphorylated BAD binds to 14-3-3 and is sequestered in the cytoplasm | − | 31, 32 |
| TSC2 | Destabilizes TSC2 and disrupts its interaction with TSC1, leading to p70S6K activation | + | 33–35 |
| Raf1 | Inhibition of Raf1 signaling | + | 36–37 |
| 14-3-3ζ | (Functional relevance remains to be determined) | − | 38 |
| Cot1/TPL2 | Increase in NF-κB-dependent transcription | − | 39 |
| IRS-1 | Positive regulation of IRS-1 function | − | 40 |
| Nur77 | Inhibits the transactivation activity of Nur77 in a nuclear receptor pathway | − | 41 |
| Gab2 | Negative regulation of mitogenic signaling | + | 42 |

[1] Laine, et al., 2002; [2]French et al., 2002; [3]Kunstle et al., 2002; [4]Laine et al., 2000; [5]Maira et al., 2001; [6]Fontana et al., 2002; [7]Basso et al., 2002a; [8]Sato et al., 2000; [9]Konishi et al., 1997; [10]Kupriyanova and Kandror, 1999; [11]Calera et al., 1998; [12]Whitmarsh et al., 2001; [13]Kim et al., 2002; [14]Fukumoto et al., 2001; [15]Konishi et al., 1995; [16]Konishi et al., 1994b; [17]Konishi et al., 1994a; [18]Paramio et al., 2001; [19]Paramio et al., 1999; [20]Jahn et al., 2002; [21]Tanaka et al., 1999; [22]Ingley and Hemmings, 2000; [23]Mitsuuchi et al., 1999; [24]Lindvall and Islam, 2002; [25]Mayo and Donner, 2001; [26]Lin et al., 2002; [27]Ogawara et al., 2002; [28]Zhou et al., 2001a; [29]Zhou et al., 2001b; [30]Lee et al., 2001; [31]Galarneau et al., 2002; [32]Datta et al., 1997; [33]Inoki et al., 2002; [34]Potter et al., 2002; [35]Dan et al., 2002; [36]Rommel et al., 1999; [37]Zimmermann and Moelling, 1999; [38]Powell et al., 2002; [39]Kane et al., 2002; [40]Paz et al., 1999; [41]Pekarsky et al., 2001; [42]Lynch and Daly, 2002.

### 5.3.6. IL-6 signalling machinery

To rationalise what is facilitating the differences seen in signalling between NDFs and KDFs, especially in serum free conditions which has not been reported, the expression of key machinery needed for the transduction of IL-6 signalling was measured; the IL-6r, co-receptor GP130 and negative regulator SOCS3.

There has been debate in the literature as to whether fibroblasts express IL-6r, with some groups suggesting that they instead rely on sIL-6r made by other cells in the environment to respond to IL-6 ligand (Spörri *et al.*, 1999; Barnes, Anderson and Moots, 2011; O'Reilly *et al.*, 2014). As we saw a signalling response in our fibroblast cultures in response to IL-6, even in the absence of serum, this suggested that fibroblasts do possess some level of functional IL-6r. Further confirming this, we saw mRNA of both full length and cleaved IL-6r in all our fibroblast cultures. Initially it was thought that the differential migration of the two IL-6r bands between fibroblasts and the THP-1 positive control during electrophoretic analysis may be due to an altered isoform. However, it is more likely to be a technical issue with the DNA dye affecting gel migration.

In 10% serum conditions there was no clear difference in mRNA levels of IL-6r, despite another study showing an increase (although not statistically significant) in IL-6r mRNA in KDFs compared to NDFs in these conditions (Ghazizadeh *et al.*, 2007). However, after serum starvation we saw a substantial increase in IL-6r after serum starvation in KDFs that was much less pronounced in NDFs which could account for the increased signalling we have observed. It was difficult to detect IL-6r at the protein level, due to the low level of expression and high auto-fluorescence of fibroblasts but we were able to see an increase in IL-6r expression in KDF in serum free conditions, which was more easily detected after permeabilising of cells, most likely due to high level of receptor recycling (Zohlnhöfer *et al.*, 1992). Reflecting the PCR results, no difference in IL-6r protein expression was seen between NDFs and KDFs in 10% serum conditions.

Despite observing the presence of cleaved IL-6r transcript that should give rise to sIL-6r, no sIL-6r was detected in 72 hour supernatants from fibroblast cultures. This is likely due to a detection limit issue, since it was possible to detect the transcript of ADAM17, which is an enzyme that is known to cleave membrane bound IL-6r from the surface to also form sIL-6r (data not shown). However, the detection of higher levels of sIL-6r in keloid tissue also suggests there may be an even greater potential for IL-6 signalling *in vivo*.

The induction of IL-6r in KDFs in serum free conditions may be physiologically relevant, given that it has been observed that IL-6r is also increased in keloid tissue (Zhang *et al.*, 2009). It

would be pertinent to investigate signalling phosphorylation with a titration of IL-6 concentrations to investigate whether this translates to a heightened capacity of KDFs to respond. Although IL-6r protein expression appeared to be very low, this may be due to the technical limitations of FACS may still be functionally significant even at low levels due to a potent effect of ligand-receptor engagement.

Intriguingly, it is also a possibility that IL-6 may be causing signalling events independent of IL-6r. This is based on observations by a group that compared the IL-6 knockout and IL-6r knockout mice and saw differences in the cutaneous wound healing process in these two different groups (McFarland-Mancini *et al.*, 2010). Both mice had delayed re-epithelisation however, IL-6 knockout mice had a much more severe wound healing deficit with delayed macrophage influx, decreased contraction and decreased fibrin clearance resulting in slow closing wounds and higher incidence of ulcers (McFarland-Mancini *et al.*, 2010). Intriguingly, another study has also found differences in these mice in a colitis model, with IL-6 deficient mice showing attenuated disease whereas IL-6r deficient mice showed no difference to the wild type mice (Sommer *et al*. 2014). This has led both authors to conclude that there may be an additional, presently unidentified receptor for IL-6. Despite the fact that the authors do not show if they have also knocked out the soluble, spliced isoform of IL-6r, an alternative receptor could be valid given the stark difference in phenotypes observed. To examine whether there is a level of signalling after IL-6 stimulation that cannot be blocked solely with Tocilizumab treatment may be a better model to answer this question.

As well as IL-6r, conventional signalling by IL-6 also requires the co-receptor GP130 which is expressed by most cell types (Heinrich *et al.*, 1998). There does not appear to be any consistent difference in GP130 expression between NDFs and KDFs in 10% serum conditions, despite an increase being reported in the literature, although again this is not statistically significant and was only examined in four biological replicates (Ghazizadeh *et al.*, 2007) . Increased GP130 expression has been seen in HSDF after trans-signalling (co-incubation with IL-6 and sIL-6r), which was not seen in NDFs (Ray *et al*., 2013). However, this conclusion is based on limited biological replicates and is not a phenomenon that is broadly acknowledged in the field. Our qPCR analysis also showed that GP130 was upregulated in 2 out of 3 KDFs after serum starvation whilst all three NDFs downregulated gp130. Increased GP130 may facilitate increased IL-6 signalling, in collaboration with increased IL-6r, as well as having an impact on signalling pathways driven by other IL-6 family members including IL-11, IL-27, LIF, OSM and CNTF (White and Stephens, 2011). GP130 can also be present in a soluble form (sgp130) and unlike sIL-6r, it acts antagonistically on IL-6 signalling (Jostock *et al.*, 2001) and has therefore been developed as a therapeutic (gp130fc fusion protein) to prevent excessive IL-6 signalling (Barkhausen *et al.*, 2011). Sgp130 was not explored in the scope of this project but could be another level of regulation in the IL-6 driven keloid pathology and there are

commercially available ELISA kits to measure this in cell culture supernatant, as with sIL-6r.SOCS3 is another important mediator of signalling, being the main "off-switch" to IL-6 signalling (Carow and Rottenberg, 2014), and in 10% serum conditions there was no clear difference in the expression of SOCS3, an important regulator of IL-6 signalling in KDFs. However, after serum starvation there was some evidence that there was less expression in KDFs which may also be related to the reduced IL-6 production we have seen in these cultures, given that IL-6 drives the transcription of SOCS3 to negative regulate its own signalling (Croker *et al.*, 2003). It would be informative to examine levels of the other main regulator of IL-6 signalling SHP2 to see if its expression is altered (Fischer *et al.*, 2004).

### 5.3.7. Effect of I-BET151 on IL-6 and downstream signalling

Given the differences we have seen in IL-6 and downstream signalling, including higher IL-6r expression, and the literature evidence for IL-6 and its signalling in pathology, the effect of BET inhibition was examined. Firstly, it was seen that BET inhibition reduced IL-6 production as has been previously reported in range of other contexts (Klein *et al*. 2014; Deeney *et al*. 2016; Garcia-Faroldi *et al*. 2017). We also have evidence that I-BET151 treatment can reduce the level of TGF-β1 induced IL-6 production (data not shown). This is likely to be regulated through well recognised mechanisms, either through inhibition of NF-κB (Belkina, Nikolajczyk and Denis, 2013) or interruption of direct interaction of BET proteins with the IL-6 promoter region through CBP (Barrett *et al.*, 2014). It would be useful in this cell system to examine the effect of I-BET151 treatment on other NF-κB regulated cytokines such as TNFα or IL-1β to determine whether this is an IL-6 specific effect as has been reported in a murine macrophage cell line (Barrett *et al.*, 2014). The effect of I-BET151 on the production of IL-17A and IL-10 in fibroblast monolayers were examined by ELISA but even the basal level of production was not detectable (data not shown).

BET inhibition also reduced signalling in a relatively short period (2 hours pre-treatment plus 30 minutes during IL-6 stimulation), especially in serum free KDFs. The ability of I-BET151 to reduce pAkt, pStat3 and pErk in KDFs suggests that BET inhibition could offer a multi-pronged approach to tackle downstream signalling pathways, which has recently been noted in ovarian cancer cells where reduction in pAkt, pErk, and pFOSL1 was observed after JQ1 treatment (Kurimchak *et al.*, 2016). There also appears to be some selectivity in its effect, with KDFs being affected more than NDFs, which could also offer a therapeutic advantage. Advantages over therapies such as IL-6r blockade with Tocilizumab that has shown success in inflammatory

arthritis (Jones and Ding, 2010), is that I-BET151 may be able to mediate downstream signalling from other ligands that may be present in disease such as MCP-1, which has been reported to be secreted by keloid CD14+ cells and cause Akt activation and proliferation in dermal fibroblasts (Liao *et al*., 2010). Further insight could be gleaned by repeating the stimulation +/- I-BET151 experiments with other keloid associated cytokines or growth factors, such as PDGF-BB, to see whether BET inhibition is indeed capable of reducing other ligand induced signalling, especially given that JQ1 and I-BET treatment had no effect on Smad signalling in lung fibroblasts after TGF-β1 stimulation (Tang *et al.*, 2013b).

### 5.3.8. Possible mechanisms of I-BET151 effect on downstream IL-6 signalling

We have confirmed the I-BET151 mediated decrease in signalling is somehow reliant on transcription through the experiment with Actinomycin D, however the mechanism is still unknown. Given the relatively short (2.5h) total time of the experiment, it is likely that the transcript/s that caused this effect has a half-life that is shorter than this time. From LPS-induced transcript half-life information on TTP-Atlas (ttp-atlas.univie.ac.at), IL6ra seems to be a very stable mRNA and is unlikely to be affected over the time course of the experiment, although the atlas data is based on macrophage experiments and there may be cell specific RNA degrading enzymes that differ in fibroblasts. Previous literature also stated, although the data is not shown, that BET inhibition using JQ1 had no effect on the expression of IL-6r in murine T cells (Mele *et al.*, 2013). We found that 2.5 hours of BET inhibition caused a small decrease in the mRNA expression of IL6r that isn't thought to be sufficient to account for the decrease seen in signalling (although it is not clear whether this translates into a change at the protein level).

The effect of BET inhibition on expression of GP130 and SOCS3 has not yet been tested. However, given the expression of GP130 on many cell types, it is unlikely to be the limiting factor in a signalling response to IL-6. Previous studies have suggested that BET inhibition either reduces SOCS3 (Ray *et al.*, 2014; Toniolo *et al.*, 2015) or has no effect on SOCS3 (Chan *et al.*, 2015) which would not provide an explanation for the reduction in signalling we have observed. An I-BET151 mediated reduction in SOCS3 would not be surprising given that IL-6 signalling drives its transcription. To further understand the mechanism behind I-BET151 mediated signalling decreases, ChIP PCR could be conducted on a range of candidate genes to see whether there is a direct interaction between BET proteins and the promoter regions of these genes.

Despite the Actinomycin D experiment results indicating that the signalling inhibition is at least partially transcriptionally mediated, there is recent evidence of a direct interaction between BRD2 and acetylated regions of STAT3, facilitating its recruitment to enhancer regions (Cheung *et al.*, 2017). However, it is not clear how binding of BRD2 to STAT3 directly relates with the level of phosphorylation it is possible that at least in part, BET inhibition may affect signalling because of the interruption of a direct Brd2-Stat3 interaction. These types of non-histone BET interactions are important and often overlooked, especially when considering the mechanistic basis of the effects seen after BET inhibition in a range of cells and disease models.

The preferential effect of BET inhibition on KDFs over NDFs is intriguing and highlights a number of possible hypotheses. Firstly, the difference in response may be due to an altered transcriptional response in these cells. It has been shown that when BRD4 is bound to super-enhancers, which may be formed during disease, it is preferentially removed from these regions following BET inhibition (Lovén *et al.*, 2014). There is strong evidence to show that chronic inflammation can drive the formation of super-enhancers (Brown *et al.*, 2014). In this way, BET inhibition therefore may 'normalise' KDF responses and downstream behaviours by targeting KDF-unique enhancer regions. Alternatively, BET proteins may be post-translationally modified in some way in KDFs that alters their function; it is known that many such modifications can occur (Wang *et al.*, 2017) but their functional consequences are not understood. Recent literature has highlighted the role of phosphorylation in the control of BRD4 function through binding to chromatin as well as other transcriptional regulators (Chiang, 2016). Lastly, there may be an altered acetylation pattern in KDFs compared to NDFs that may facilitate differential BET protein binding as this could be explored further through acetylome analysis.

### 5.3.9. Possible functional relevance of increased IL-6 signalling in KDFs

As well as understanding the mechanism of reduced signalling by I-BET151 it is highly important to understand the functional relevance of this and whether this may indeed be linked to other cell changes that have been seen in this project such as decreased proliferation, α-SMA expression, contraction and protease activity. However, gauging the link between a signalling response and a cell behaviour is not clear cut, for example it has been shown that KDFs have a robust activation of Erk signalling, comparable to that of NDFs, after EGF stimulation, however they show limited downstream activity in the form of a migratory response (Satish *et al.*, 2004). To understand this better, future experiments should first focus on characterising the response of

NDFs and KDFs to IL-6 stimulation, especially in a serum free context, with a functional read-out and then identifying the importance of the individual pathways (Stat3, Erk and Akt) in this response with pharmacological inhibitors.

It is not yet clear from current literature whether IL-6 is causing disease relevant cell behaviour in keloids, or whether it is simply a side effect of the chronic environment and simply serves as a biomarker. However, Ponceau Red staining of serum free IL-6 stimulation experiment western blots indicated a unique protein response in KDFs, with induction of an unidentified protein at ~70kDa (data not shown) suggesting these cells are responding differently at a protein level. It would also be highly informative to characterise the effect of IL-6 stimulation on NDFs vs KDFs with a transcriptomic and proteomic approach. RNA-seq and discovery mass-spectrometry on NDFs and KDFs +/- IL-6 stimulation in serum free conditions would be hugely useful tools to unravel a potentially abnormal response of KDFs, that may also be shared with other inflammatory or fibrotic diseases.

A publication in 2009 isolated colony forming 'precursor' cells from keloid scar and normal skin tissue, which could be interpreted as fibroblasts, and found that in low 1% serum conditions both cell types proliferated to the same extent in response to IL-6 stimulation shown by BrdU staining (Zhang *et al.*, 2009). However, when these cells were incorporated into a hydrogel with IL-6 before transplantation into immunocompromised mice, the keloid derived cell gel grew faster than the normal skin derived equivalent. They also found that the keloid cells produced stereotypically thick collagen fibrils when examined histologically (Zhang *et al.*, 2009) although the data in the supplementary materials supporting this statement was difficult to interpret. When naïve T cells were added to this system, the growth of the gels was further increased (Zhang *et al.*, 2009). This demonstrates the advantages of increasing complexity in culture models to better understand the importance of IL-6 in the *in vivo* environment, which may provide even more insight into the relevance of the intrinsic differences we have seen in IL-6 and downstream signalling in NDFs vs KDFs.

# Chapter 6: Overall conclusions and future direction

## 6.1. Principal findings

In order to best represent the contribution of this thesis to the technical field, a summary of the main findings of this project, including those of particularly novelty are represented in Table 6.1. Those findings that were novel, rather than reaffirming previous literature reports, such as simply the anti-proliferative effect of BET inhibition, are boldened in the table. Those findings that are deemed as of particular importance or significance to the field, whether that be to the keloid or the BET protein field, are further highlighted by underlining.

***Table 6.1*** **- Summary of thesis contributions.**

| Chapter | Key findings |
|---|---|
| **3** – Characterisation of BET protein expression in keloid scars | • **Brd2 appears to be increased at the mRNA and protein level in a subset of keloid tissue samples.**<br>• **<u>Possible expression of a differential 60kDa isoform of Brd2 that may have alternate functions.</u>**<br>• Brd2, Brd3 and Brd4 are all expressed in dermal fibroblasts with **no difference between NDF and KDF in standard culturing conditions**.<br>• **Inflammatory factors including TNFα and LPS appears to induce Brd2 expression in NDF.** |
| **4** – Evaluating the effect of BET protein inhibition on disease associated cell behaviours | I-BET151 treatment<br>• Decreases **dermal fibroblast** proliferation with **<u>possible increased sensitivity in KDFs compared to NDFs</u>**.<br>• Does not affect collagen expression or have any clear effect on contraction despite decreasing ACTA2 expression.<br>• Appears to decrease overall MMP levels and **<u>protease activity in fibroblasts and in whole tissue.</u>** |
| **5** – Investigating the effect of BET protein inhibition on differential IL-6 signalling in keloid scars | • Confirmed increased IL-6 secretion in KDFs and keloid tissue reported in the literature.<br>• Confirmed increased pStat3 in KDFs but also showed **<u>increased pAkt and pErk1/2 compared to NDFs.</u>**<br>• **<u>Striking induction of pAkt in KDFs in serum free conditions after IL-6 conditions not seen in NDFs.</u>**<br>• **<u>Confirmed presence of IL-6r at mRNA and protein level in dermal fibroblasts</u>** |

*Novel findings not previously reported are boldened, and those also underlined are deemed as particularly important to the field.*

Keloid scars are currently an unmet clinical need, growing invasively outside the original wound margin, often becoming very itchy and painful for the patient and frequently unresponsive to current lines of treatment (Gauglitz *et al.*, 2011). In this work, we have confirmed what has been previously reported in keloids, including increased levels of the matrix transcripts COL1A1, COL3A1 and FN compared to normal skin, which are typical scar components (Xue and Jackson, 2015). However, we have also seen evidence in this project and through other work in the lab, that keloid scarring is a distinct form of scarring. We have shown that transcript levels of α-SMA are much lower in keloid scar than in normal skin, which is contrary to what has been reported in many other fibroses, where increased α-SMA levels are indicative of myofibroblasts (Hinz *et al.*, 2007). Interestingly, unpublished data from the lab suggests there is a unique and abnormal ECM production in keloid scars rather than just over-deposition of typical fibrillar collagens.

We have also seen substantial heterogeneity between patients, both in their tissue characteristics and in isolated fibroblasts, such as the variable levels of IL-6 production and basal signalling. Clinically, patients differ greatly; for example, in our patient collections, one female patient had a single non-itchy, non-painful keloid with no family history, whereas one male patient had multiple, itchy and painful scars and had a family history of keloids. The lack of stratification of these samples is not only something that is absent from scientific research in this area but also within the clinic; many patients respond well to hydrocortisone steroid injections whereas others do not (Ud-Din *et al.*, 2013), and the basis for this is not fully confirmed. In the future, better patient stratification may help inform treatment strategies.

Unexpectedly, we saw many common cell characteristics between NDFs and KDFs, such as proliferation; however, reports of KDF hyper-proliferation in the literature are also variable. However striking differences were seen in IL-6 secretion and downstream signalling and importantly demonstrates that there are some intrinsic characteristics in keloid dermal fibroblasts that are still retained despite *in vitro* culturing. These differences that we have found to date emerged when the culture conditions were simplified with removal of serum. Colleagues have also found through transcriptional profiling experiments that in order to see differences between healthy and disease cells, serum levels in culture need to be reduced to at least 0.1% (personal communications, Dr Christina Phillippeos).

For the first time, serum starvation revealed striking differences in downstream IL-6 signalling in KDFs; particularly, IL-6 strongly induced pAkt, which was not seen in NDFs. Although the functional consequences of this are not yet understood, it does demonstrate a distinct phenotype that is retained in these cells in culture that may be due to an epigenetic ‘memory’. High levels of IL-6 and differential downstream signalling are anticipated to be of relevance in keloid scars, although direct experimental investigation of this was outside the scope of this project but still

highly relevant given its proinflammatory reputation and marked reduction after I-BET151 treatment. From ongoing experiments in the lab, it appears it is unlikely that the differential signalling is responsible for driving a proliferative response, as there is no keloid specific proliferative response to IL-6 stimulation in serum free conditions. We are currently exploring whether this signalling could be driving other cell behaviours including survival, migration and fibrotic gene expression.

*In vivo* it has been postulated that IL-6 signalling could be linked to endothelial dysfunction as described in (Ogawa & Akaishi, 2016; Wassmann *et al*., 2004) which may drive inflammation through vessel permeability. IL-6 could also be a mediator of cellular contraction in this model; although it is thought that IL-6 does not directly drive fibroblast contractility, as shown in a scarring trachoma study, it is thought to cause the activation of macrophages which could in turn cause increased contractility (Kechagia *et al.*, 2016).

A large part of this project was investigating BET proteins and the potential therapeutic value of BET inhibition by modifying keloid associated cell behaviours including IL-6 and downstream signalling. Patient heterogeneity was also seen in the expression of BET proteins in tissue, however higher expression of BRD2 in keloid tissue was observed convincingly at the mRNA level and in a subset of patients at the protein level. IHC analysis suggested that fibroblasts were strongly positive for BRD2 and so further analysis focussed on the role of BET proteins in fibroblasts. In further investigations, NDFs and KDFs did not show any obvious differences in BET mRNA or protein expression, which may be due to a limitation of our culturing system, although it was found that some inflammatory stimuli, such as TNFα could increase BRD2 expression.

Analysis of BRD2 protein by western blot indicated a band at 60kDa, which may coincide with a predicted isoform that lacks the NLS and NET domains with a likely impact on protein function. Although we could not confirm the identity and sequence of this band by alternative methods in the scope of this project, it does give some possible explanation for differential BET protein function in normal skin vs keloid tissue. The possible existence of this shorter isoform is especially interesting given that a short BRD4 isoform has reported to be pro-metastatic due to more promiscuous histone binding (Alsarraj *et al.*, 2013) and this warrants further investigation.

BET inhibitors have been progressing in a range of clinical trials and there is growing interest in the application of these treatments to organ fibrosis (Stratton, Haldar and McKinsey, 2017). The first evidence within the skin has now shown BRD4 knockdown decreases myofibroblast differentiation (Ijaz *et al.*, 2017) and much of the data in this project would add to the body of evidence that BET inhibition could be beneficial in fibrotic scarring in the skin, including for keloids. We have confirmed previously reported effects of BET inhibition that were observed in other cell systems, including decreases in proliferation and a decrease in the pro-

inflammatory cytokine IL-6. However, we have added knowledge to the field with the observation of additional novel effects of BET inhibition, including a reduction in contraction in a collagen gel model, and a reduction in protease activity. These findings may be applicable to other diseases including cancers; for example, I-BET151 could offer a solution to the increase in MMP activity in the cancer microenvironment, which drives the invasive migratory behaviour of cells and subsequent metastasis (Sternlicht and Werb, 2009).

The mechanisms of the observed effects of I-BET151 on cell behaviour, especially the KDF-specific inhibition of downstream IL-6 signalling, are difficult to determine without large scale analyses such as ChIP-Seq to identify important BET protein binding patterns. Transcriptional and epigenetic datasets are available from associated BET inhibition and BET siRNA studies, especially for Brd4, which is known to be associated with super enhancers that have particular importance in cancer (Lovén *et al.*, 2014). The indication of heightened sensitivity of KDFs to I-BET151, in terms of proliferation and IL-6 signalling, may not be related to levels of BET proteins but instead due to differential binding patterns and activity. Very little is known about the regulation of BET protein activity, with some new literature emerging suggesting that phosphorylation of BRD4 can affect its activity (Wu *et al* 2013) with hyper-phosphorylated BRD4, modulated by casein kinase II (CK2), recently being associated with NUT midline carcinoma (Wang *et al.*, 2017).

Interestingly, cell specific I-BET151 effects were unravelled in the signalling experiments, where I-BET151 specifically decreased signalling only in KDFs in serum free conditions, without effect on signalling in NDFs in the same conditions. This echoes findings from GSK (personal communications), whereby BET inhibitor treatment appears to have specific effects on disease cells to “normalise” their profile and behaviour to a healthy level.

## 6.2. Project limitations

One limitation of the work is our 'normal' control tissue. Due to ethical reasons, it is not possible for us to isolate normal, unaffected skin from keloid patients due to the risk of new keloid formation. Occasionally samples were received with a small normal skin border, but it is also debatable about how 'normal' this tissue really is. Our normal skin samples tended to be derived from Caucasian patients aged 40-50+ whereas our keloid scar samples tended to be from younger patients with darker skin, which is relevant given that both age and ethnicity are known to influence skin biology (Rawling, 2006; McGrath, Robinson and Binder, 2012). An appropriate normal scar was difficult to come by; for the IHC analysis we used scars from melanoma re-excision patients at ~6 weeks which may be slightly less normal due to their association with cancerous tissue and are also small and clinically precious. Older scars may be accessible from scar revision surgeries, but these are likely to be relatively resolved with a low cellular content and cleared inflammation (Desmoulière *et al.*, 1995) unlike keloid scars that are often still in active inflammation and growth. In addition, this project did not discriminate keloids according to location of sampling, with recent literature making clear that keloids are spatially diverse and may have different cellular activity in the actively growing edge vs the hypoxic fibrotic core (Jumper *et al.*, 2017).

In addition, in this project, and in the field in general, there is a limitation in the lack of stratification of keloid tissue. There is increasing awareness of spatial variability within keloids at the actively growing edge region for example, compared to the hypoxic core. Fibroblasts may retain some epigenetic memory of their environment and therefore cells from the edge region could potentially be more metabolically active, more proliferative, more contractile and more proteolytically active than those found at the core. This limitation came to light in particular during the analysis of ACTA2 expression in keloid tissue, where although the expression was somewhat anticipated to be higher than normal skin, it was found to be lower compared to normal skin. This is likely due to these tissue samples originating from the core region of keloid where tension and myofibroblast activation is likely to be lower. As such, it is important to evaluate potential effects of BET inhibition at a spatial, as well as whole keloid, level to ensure that the drug does not cause a decrease in MMPs at the core for example, when an increase in MMP, in order to clear the fibrotic matrix deposition, is desired.

A factor that is limiting work in the entire human dermal fibroblast field is the lack of a unique cell surface marker. Part of this problem is that isolated fibroblast populations are often treated as a single population as we have done in this project. However, growing evidence is showing that fibroblasts are heterogeneous, with subpopulations that may have different functions in wound repair. It has been noted for several decades that site-matched fibroblasts from the

papillary and reticular dermis have different characteristics. Papillary fibroblasts appear to have higher levels of proliferation, collagen deposition, growth factor production and a distinct gene expression signature in comparison to reticular fibroblasts (Harper and Grove, 1979; Tajima and Pinnell, 1981; Sorrell, Baber and Caplan, 2004; Janson *et al.*, 2012). More recently, it has been shown in mice that these two types of fibroblasts respond differently in wounding. A lineage of fibroblasts which make up the lower dermis, including the reticular layer and hypodermis, are responsible for the first wave of dermal repair and are not able to support formation of new hair follicles. The upper lineage of fibroblasts are only recruited later in wound repair, during re-epithelisation (Driskell *et al.*, 2013). Currently, most of the understanding of fibroblast heterogeneity is restricted to mouse models but work is ongoing to characterise such functional subpopulations in humans and their contribution in homeostasis and in wound response. Given that we do not yet know the origin of keloid fibroblasts, increasing understanding of these subpopulations and availability of markers to define them will allow us to investigate whether keloid fibroblasts may be an expansion of a specific subpopulation.

Another limitation of our model is that fibroblast behaviour is somewhat abnormal *in vitro.* It is increasingly appreciated that the artificial 2D plastic *in vitro* environment may cause drastic changes in cell morphology and behaviour, including gene expression (David, Sayer and Sarkar-Tyson, 2014; Yeung *et al.*, 2015). The rigid plastic surface on which cells are cultured in plates or tissue culture flasks generates unnatural tension which would not be experienced *in vivo* (Wells, 2008). In fact, a transcriptomic study demonstrated that exposure of fibroblasts to serum induced a wound-like and cancer-like response (Chang *et al.*, 2004). There are also many factors that are still absent from the system, including ECM and the presence of other important cell players such as immune cells or keratinocytes (Werner, Krieg and Smola, 2007). Given the plasticity of dermal fibroblasts, which is utilised as an advantage in regenerative medicine and stem cell research, it is likely that paracrine factors from other cells can induce significant expression changes (Slany *et al.*, 2014).

A comprehensive characterisation of keloid tissue would be hugely beneficial in understanding the cellular composition of keloids. A single paper has taken an IHC approach to identify these cells, but have found that almost all immune cell subsets are increased in keloid scars (Bagabir *et al.*, 2012a). This project attempted to isolate cells directly from tissue for *ex vivo* analysis by FACS however this was technically challenging with high auto-fluorescence, low digestion efficacy and lack of fibroblast marker. A better understanding of the cellular composition would also aid in creating better models of keloids, which are currently very limited, both through tissue engineering techniques and animal models, which is hampering all research into the early stage of keloid development. The transplantation of human keloid tissue to immune compromised mice is currently the best used in vivo model of keloids, but are not wholly suitable, as it is likely that immune cells have a strong influence on the disease, and it does not

give information on the initiating events of keloid formation (van den Broek *et al.*, 2014). There has been a number of reviews in recent years which have summarised the current research status of appropriate scar models including for keloids (Williams, Herndon and Branski, 2014; Suttho *et al.*, 2017). The consensus is that the modelling of fibrotic scars is extremely complicated, given their complex biology and immune cell influence. Keloid-like tumours can form on the lower leg of some horses although they are not epithelialised in the same way as keloids (Theoret *et al.*, 2013). Duroc pigs also produce a hypertrophic version of scarring, but this is still likely to be a distinct pathology in comparison to keloid scars (Zhu *et al.*, 2003).

The *ex vivo* biopsy tissue culture model was initially anticipated to be a useful tool to evaluate the effect of I-BET151 on all the important cell types in keloid tissue, using qPCR, western blot or IHC to analyse markers of disease such as Collagen I, markers of proliferation such as Ki67 and markers of inflammation such as IL-6. An extensive collection of these models was formed, however upon IHC staining there was a complete absence of Ki67 unlike freshly isolated tissue which showed strong positivity in the basal keratinocyte layer and scattered fibroblast positivity in the upper dermis (data not shown). This indicated that the tissue health was not optimal so the decision was made not to pursue further analysis of these samples. In revisiting the original publication of this model, minimal evidence was given for tissue health, as data for the week 2 time point indicated over 25% of cells were Ki67 positivity which is surprisingly high depending on the location within the tissue, whereas over 15% of cells were TUNEL positive indicating apoptosis (Bagabir *et al*. 2012b) which would have not allowed for the extraction of good quality RNA.

RNA extraction from fibrous tissue is notoriously difficult and despite efforts to optimise homogenisation and extraction protocols for keloid tissue, RINs (a measure of RNA integrity) were still somewhat variable. A small pilot study indicated that a 1 hour incubation in dispase solution at 37°C to remove the epidermal layer of the tissue caused a substantial decrease in RIN so whole tissue was used for this work. The variable RINs of keloid tissue may be due to fibrous matrix not allowing for the penetration of RNAlater preservation solution, despite only being a 4mm diameter biopsy. In future, it may be beneficial to finely dice tissue before preservation in RNAlater solution to improve preservation and this may also assist in achieving better homogenisation and RNA yield. An investigation of RNA quality in a tissue bank reported that fatty, fibrous or necrotic samples had relatively low RINs (Kap *et al.*, 2014). The average RIN for most tissue types was between 7 and 8, whilst skin tissue had an average of 4.95 (Kap *et al.*, 2014) demonstrating the technical challenge of isolating high integrity RNA from fibrous skin samples. Despite this, there are varying reports on the importance of RNA integrity in qPCR analysis and indeed the accuracy of Bioanalyser-calculated RIN numbers as a measure of integrity. A review published in 2006 stated “there are no statistically confirmed studies at which threshold RNA integrity is useless for quantitative downstream applications”

(Fleige and Pfaffl, 2006). This study found that RNA integrity did impact cycle threshold values, but this was counteracted by normalisation to a reference gene.

## 6.3. Future directions

There is a variety of possible research avenues that have arisen from this project and those deemed of the highest importance are described below.

Firstly, knockdown of specific BET family members would allow the investigation of the contribution of each BET protein to fibroblast biology. There is increasing awareness that BET proteins may have differing roles in health and disease. It has been shown that BRD2 and BRD4 have differing binding patterns and roles in T cells to drive Th17 differentiation (Cheung *et al.*, 2017). Equally, in multiple disease models, different effects of BET inhibitors vs individual knockdowns have been observed. For example, BRD4 knockdown in TGF-β1 treated dermal fibroblasts could stop myofibroblast differentiation in the form of α-SMA expression, whereas JQ1 treatment achieved this whilst also preventing the production of fibronectin and collagen 1a1 (Ijaz *et al.*, 2017), suggesting that another family member (possibly Brd2 based on our own observations) is responsible for the regulation of these genes. Due to technical challenges and the time constraints of this project, it was not possible to overexpress Brd2 in dermal fibroblasts to assess functional importance. However, there is an indication from the literature that increased expression of BRD2 may cause acceleration of cell cycling in fibroblasts (Sinha, Faller and Denis, 2005).

Secondly, it is important to note that all effects seen in this project after I-BET151 treatment are due to changes to bromodomain activity and therefore acetylated lysine binding, but not overall BET protein function. Given that there are other functional domains that may have important biological roles, it would be informative to better understand the role of other functional domains. A recent publication by the Bradner group demonstrated a new tool wherein JQ1 was conjugated to a phthalimide compound in order to target BET proteins for proteasome degradation (Winter *et al.*, 2015). This could, therefore, be compared to a conventional BET inhibitor to evaluate the importance of non-bromodomain regions of BET proteins. In this project, we found a specific overexpression of BRD2 and a possible expression of a differential 60kDa isoform lacking the NLS and NET domains. Confirmation of the presence of this isoform by pull down and mass spectrometry was unsuccessful, but the use of an immunoprecipitation validated BRD2 antibody could allow this in the future. Transfection of cells with individual functional domains, or indeed the BRD2 short isoform, would also offer an approach to explore functionality in fibroblasts.

To better understand the mechanism of action of BET proteins in our system, ChIP-seq, or the recently described click-seq (Tyler *et al.*, 2017), could also be conducted in our fibroblast cultures to analyse interactions between BET proteins and DNA. Existing datasets could be mined, however as these proteins are known to have very cell-specific and stimuli-specific binding this may only be slightly helpful. In addition, immunoprecipitation and mass spectrometry studies would also be helpful to investigate direct interactions between BETs and non-histone proteins, especially given the cytoplasmic presence we have seen in BRD2 staining of keloid tissue and fibroblasts. Gaining a better understanding of the mechanisms of action of I-BET151 is important for the progression of these compounds into the clinic.

Lastly, another important avenue for future investigation is to better characterise the differential response to IL-6 in KDFs, especially in terms of the drastic induction of Akt signalling we have seen in serum free media. Firstly, we would like to examine the dynamics of the signalling response to IL-6 rather than the single timepoint of 30 minutes that has been examined in this project. Then we would like to understand the functional consequence of IL-6 stimulation in serum free media by looking at a range of read-outs, to explore whether there is a keloid-specific cellular response to IL-6. Ongoing work suggests that there is no difference in the proliferative response to IL-6 in NDFs or KDFs, but we plan to also look at read-outs of other behaviours including apoptosis/survival, migration, and matrix production. Once a functional difference is identified, the contribution of Akt signalling could be explored using inhibitors of the Akt pathway, such as MK2206 (Hirai *et al.*, 2010).

In conclusion, the observations we have made in this project have added further evidence for an anti-proliferative and anti-inflammatory effect of BET inhibitors, but also offers new *in vitro* information that BET inhibition decreases contractile and proteolytic activity of dermal fibroblasts, and a disease specific effect on IL-6 downstream signalling, which may have relevance in the treatment of keloids.

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# Chapter 7: Appendix

## 7.1. Example patient information sheet

**Keloid Information Sheet for Researchers**

| | |
|---|---|
| **Patient Identification Number for this study (YYMMDDa/b/c...)** | 20150305 a |
| **Sex** | M (F) |
| **Date of Birth** | 14/11/97 |
| **Site of Scar** | (L) earlobe. |
| **Age of scar (approx. is fine)** | 5 years. |
| **Single or multiple scars** | Single (Multiple) |
| **Family history** | (Yes). No |
| **Description (e.g. hard/soft/itchy/ painful/coloured)** | Itchy, hard. |
| **Previous interventions** | Haelan tape. |
| **Date of last steroid injection** | |
| **Steroid-responsive?** | Yes (No). Minimal short term |
| **Location of biopsy within scar (e.g. edge, full width, full depth...)** | Full width |

***Figure 7.1*- Completed example patient information sheet**

## 7.2. BRD2 immunoprecipitation

In an attempt to identify the 60kDa band that appeared in tissue lysate western blots when probed for BRD2, as well as any other keloid unique bands that may indicate a unique direct protein BRD2 interaction, an immunoprecipitation was undertaken using the same GSK polyclonal antibody. This was conducted on two normal skin and one keloid scar lysate samples before silver staining to visualise pulldown proteins which appeared to show unique bands (**Figure 7.2**). However, upon running the antibody alone and silver staining, it was clear that this was an antibody specific band (**Figure 7.2**). This suggested that the antibody was not suitable for immunoprecipitation and due to time constraints it was not possible to follow this up with an alternative antibody.

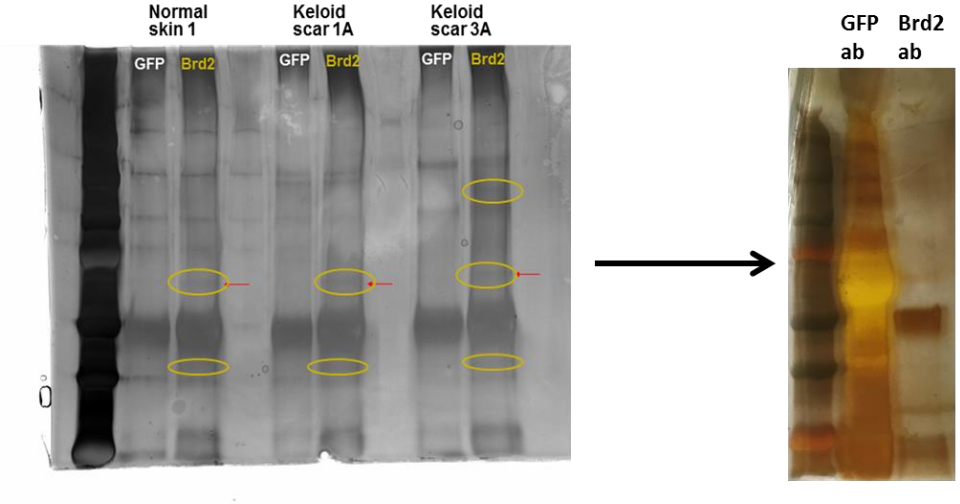



***Figure 7.2* - Silver staining of BRD2 immunoprecipitation.** BioRad SureBead IP system was used to pull down three tissue lysates using the GSK BRD2 polyclonal antibody and an irrelevant GFP control antibody. Briefly the magnetised beads were coated with antibody, washed and incubated with 25µg of tissue lysate in 500µl total volume of RIPA buffer, following more washes beads were washed in elution buffer to elute the antibody bound to target protein. This elution buffer was run on an SDS gel as described in Materials & Methods and then Silver Stained. The gel appeared to show unique bands in the BRD2 specific lanes, however after running antibody alone and silver staining it was clear that these were antibody specific rather than indicative of target protein at these molecule weights.

## 7.3. 5’ RACE-PCR for BRD2 isoform analysis

In order to identify possible differential BRD2 isoforms that may have interesting cellular functions, two approaches were attempted.

Firstly, 5’ RACE-PCR (also known as one sided or anchored PCR) was conducted using the FirstChoice™ RLM-RACE Kit (cat no. AM1700, ThermoFisher Scientific). Two internal primers were designed at the most 5’region which was common to all known transcripts as depicted in (**Figure 7.3A**)**.** A forward primer was also designed as a positive control to check the success of the reverse primers during optimisation (**Figure 7.3B**). The primers were tested in a PCR using a *BRD2* plasmid supplied by GlaxoSmithKline and showed two distinct bands of the correct size indicating effective primer design (**Figure 7.3D**).

RNA was then prepared from a NDF as depicted (**Figure 7.4A**) firstly by treatment with calf intestinal alkaline phosphatase (CIP) to remove 5’$PO_4$ to ensure no degraded mRNA or other types of RNA are amplified. Then the RNA is treated with tobacco acid pyrophosphatase (TAP) which de-caps full length mRNA to leave a mono-phosphate that a kit designed 5’ RACE adapter can be ligated onto. After ligation, reverse transcription was conducted as per usual protocols using random primers.

The resulting cDNA was then used in two rounds of PCR, firstly using the outer primer set (one user designed outer reverse primer and one kit supplied outer forward primer). 5µl was kept for electrophoretic analysis and 2.5µl was used as the template cDNA for next round of PCR using the inner primer set.

5µl of each round of PCR, including the NTC were run on a 2% agarose gel at 100V until good separation of the ladder. A single band was visualised in the second round of PCR but upon sequencing this did not appear to be a true amplicon (**Figure 7.4B**).

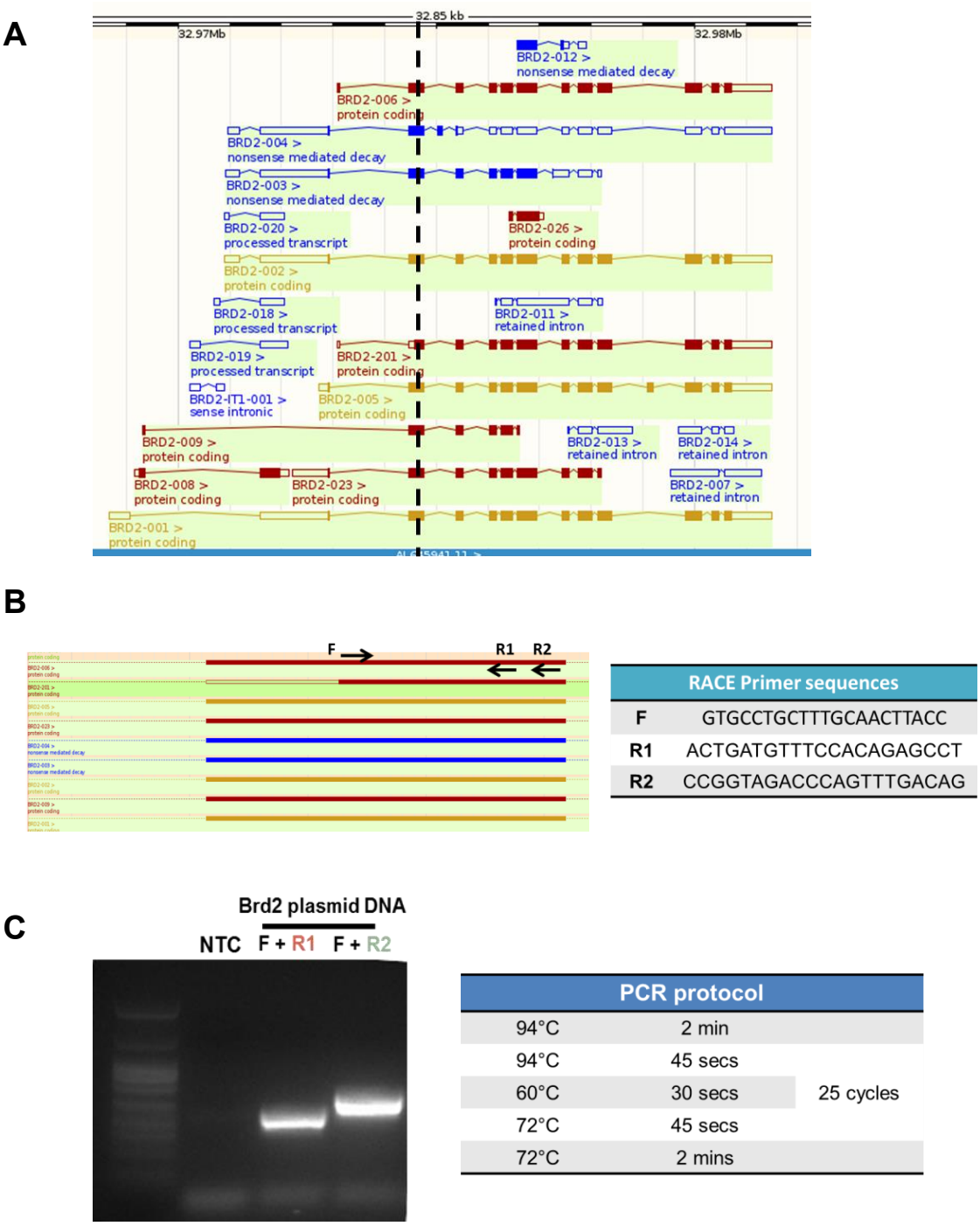
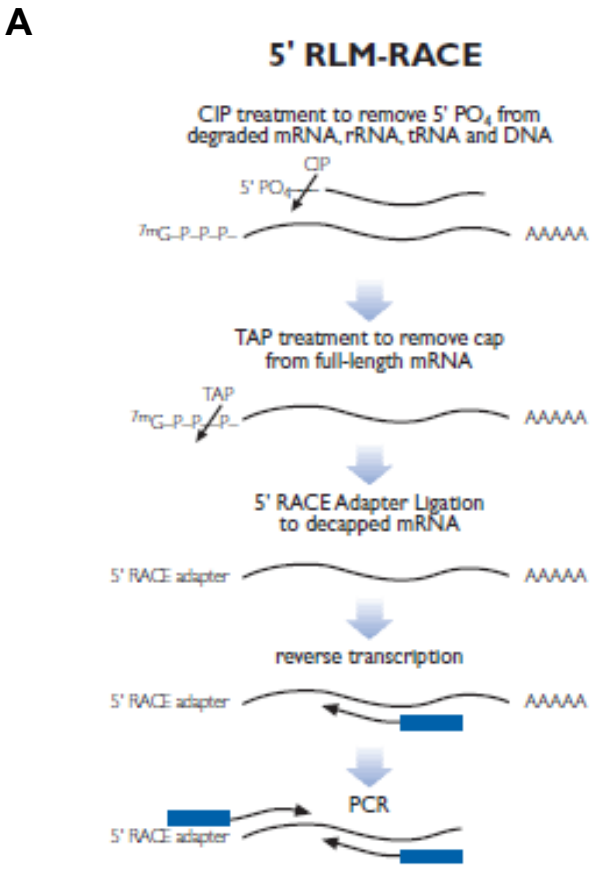
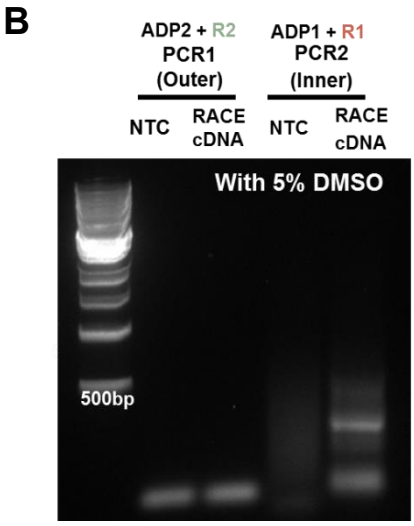



***Figure 7.3* - 5' RACE PCR primer design.** (**A**) Primers were designed at the first shared exon (exon 2) for the majority of BRD2 transcripts as denoted by the dashed line. (**B**) A forward primer (F) was designed as a positive control and two nested reverse primers (R1 and R2) were designed 133bp and 184bp upstream of F with sequences shown. (**C**) RT-PCR was run using a Brd2 plasmid as template showing specific amplification of a 133bp and 184bp transcript from the two nested reverse primers (R1 and R2) with the forward primer (F) with the cycling conditions shown with products run on a 2% agarose gel.




| PCR protocol | | |
|---|---|---|
| 94°C | 2 min | |
| 94°C | 45 secs | |
| 60°C | 45 secs | 35 cycles |
| 72°C | 5 mins | |
| 72°C | 5 mins | |

***Figure 7.4*** **- 5'RACE PCR on fibroblast RNA.** (**A)** RNA was prepared for 5'RACE RNA as shown. mRNA was decapped, ligated with the 5' RACE adapter before reverse transcription as previously described in Methods & Materials. (**B**) Prepared cDNA was then used for two successive rounds of PCR, the first 'outer' round using the outer primer set (outer kit supplied primer specific for the ligated 5' RACE adapter + R2) and the second 'inner' round using the inner primer set (inner kit supplied prime specific for the ligated 5' RACE adapter + R1) under the cycling conditions as shown, with the addition of 5% DMSO into each PCR reaction to reduce secondary structures. No bands were seen following the first outer round of PCR however a small sized band of ~150bp was seen after the second inner round.

## 7.4. qPCR for BRD2 isoforms

Another approach was then attempted to identify BRD2 isoform transcripts, using qPCR primers designed with the help of Catriona Sharp, GSK. Primers were designed to amplify specific Brd2 transcripts (**Figure 3.12**), however it was only possible to design unique primers for single transcripts for BRD2-001 and BRD2-002. The rest of the primer sets amplified different combinations of the transcript options (**Table 7.1**).

The three primers were selected for each set (shown in blue in **Table 7.2**) and these were used for qPCR on n=4 normal skin and keloid scar tissue RNA isolations alongside the reference gene B2M. The best primer for each set out of the selected three, chosen on the basis of standard curve $R^2$ and primer efficiency score (calculated by the slope of the standard curve) was selected and relative mRNA expression was calculated for each sample using the standard curve method and normalising expression to the reference gene B2M (**Figure 7.5**).

Relative expression results were conflicting, as primers that amplified a single transcript e.g. Set 1 showed higher relative expression than primers that amplified many different transcripts, including this single transcript e.g. Set 3. This was deemed to be a primer efficiency issue and therefore hindered our ability to make a comparative analysis of primer sets as planned.

***Table 7.1*** **- Summary of BRD2 isoform specificity for each primer set.**

| | **BRD2 Isoform Transcripts** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **001** | **002** | **005** | **006** | **009** | **201** | **023** | **026** |
| **Set 1** | ✓ | | | | | | | |
| **Set 2** | | ✓ | | | | | | |
| **Set 3** | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | |
| **Set 4** | ✓ | ✓ | | | | | ✓ | |
| **Set 5** | ✓ | ✓ | ✓ | | | | ✓ | |
| **Set 6** | | | | ✓ | | ✓ | | |

***Table 7.2* - List of BRD2 isoform primers.** Designed by Catriona Sharp (GSK) with 5 primer pairs to amplify the same set of transcripts with some repetition of forward or reverse primers.

| Set | Pair | Forward | Reverse |
|---|---|---|---|
| **1** | **1** | **GGGATAGGAAGCTGGGGATA** | **CGAAACCCAGAGCGCTATAA** |
| 1 | 2 | AGGAAGCTGGGGATATGGAC | CGAAACCCAGAGCGCTATAA |
| **1** | **3** | **GGGATAGGAAGCTGGGGATA** | **CTATGTCCCGAAACCCAGAG** |
| 1 | 4 | TAGGAAGCTGGGGATATGGA | CGAAACCCAGAGCGCTATAA |
| **1** | **5** | **TAGGAAGCTGGGGATATGGA** | **CTATGTCCCGAAACCCAGAG** |
| 2 | 1 | GGGGAGGGTTTTTCCCTTA | GGCTCAGAGCAGAAAAAGCA |
| **2** | **2** | **GGGGAGGGTTTTTCCCTTA** | **GCTCAGAGCAGAAAAAGCAAG** |
| **2** | **3** | **GGGGAGGGTTTTTCCCTTA** | **GCTCAGAGCAGAAAAAGCAA** |
| **2** | **4** | **ATACGGGTGTGCCTTTGG** | **AGTCCTGGGAGGGATGACTT** |
| 2 | 5 | GGGGAGGGTTTTTCCCTTA | CAGAGCAGAAAAAGCAAGTCC |
| **3** | **1** | **GAGGTGTCCAATCCCAAAAA** | **AGGTATTGCAGCTGGTTGGT** |
| **3** | **2** | **GAGGTGTCCAATCCCAAAAA** | **TTTCCACAGAGCCTTCATCA** |
| **3** | **3** | **AAAGCCAGGACGAGTTACCA** | **TTTCCACAGAGCCTTCATCA** |
| 3 | 4 | AAGCCAGGACGAGTTACCAA | TTTCCACAGAGCCTTCATCA |
| 3 | 5 | GAGGTGTCCAATCCCAAAAA | CATGCGAACTGATGTTTCCA |
| 4 | 1 | GAACGAGCTGGAGGATTCTG | CGGATGGAGGTGGATTGTAA |
| **4** | **2** | **GAATCCCTGCAGACCAACAG** | **GATCTCAGACACCGTCGTCA** |
| **4** | **3** | **AACAGCGGGCTATATTGACG** | **GGCAACTAAGGAGGGACTGA** |
| **4** | **4** | **CCCGACAAGAAGAGGGAATC** | **CGTCAATATAGCCCGCTGTT** |
| 4 | 5 | AACAGCGGGCTATATTGACG | GGGCAACTAAGGAGGGACTG |
| **5** | **1** | **ACTTAGCGGGTTATGCTGGA** | **ATCTTGACCGCAAGGAACC** |
| 5 | 2 | CTTAGCGGGTTATGCTGGAC | ATCTTGACCGCAAGGAACC |
| **5** | **3** | **CCAACTTAGCGGGTTATGCT** | **ATCTTGACCGCAAGGAACC** |
| 5 | 4 | ACTTAGCGGGTTATGCTGGA | CATCTTGACCGCAAGGAAC |
| **5** | **5** | **CTTAGCGGGTTATGCTGGAC** | **CATCTTGACCGCAAGGAAC** |
| **6** | **1** | **GAGGTGTCCAATCCCAAAAA** | **AGGTATTGCAGCTGGTTGGT** |
| **6** | **2** | **GAGGTGTCCAATCCCAAAAA** | **TTTCCACAGAGCCTTCATCA** |
| **6** | **3** | **AAAGCCAGGACGAGTTACCA** | **TTTCCACAGAGCCTTCATCA** |
| 6 | 4 | AAGCCAGGACGAGTTACCAA | TTTCCACAGAGCCTTCATCA |
| 6 | 5 | GAGGTGTCCAATCCCAAAAA | CATGCGAACTGATGTTTCCA |

Primers highlighted in blue were selected for ongoing analysis.

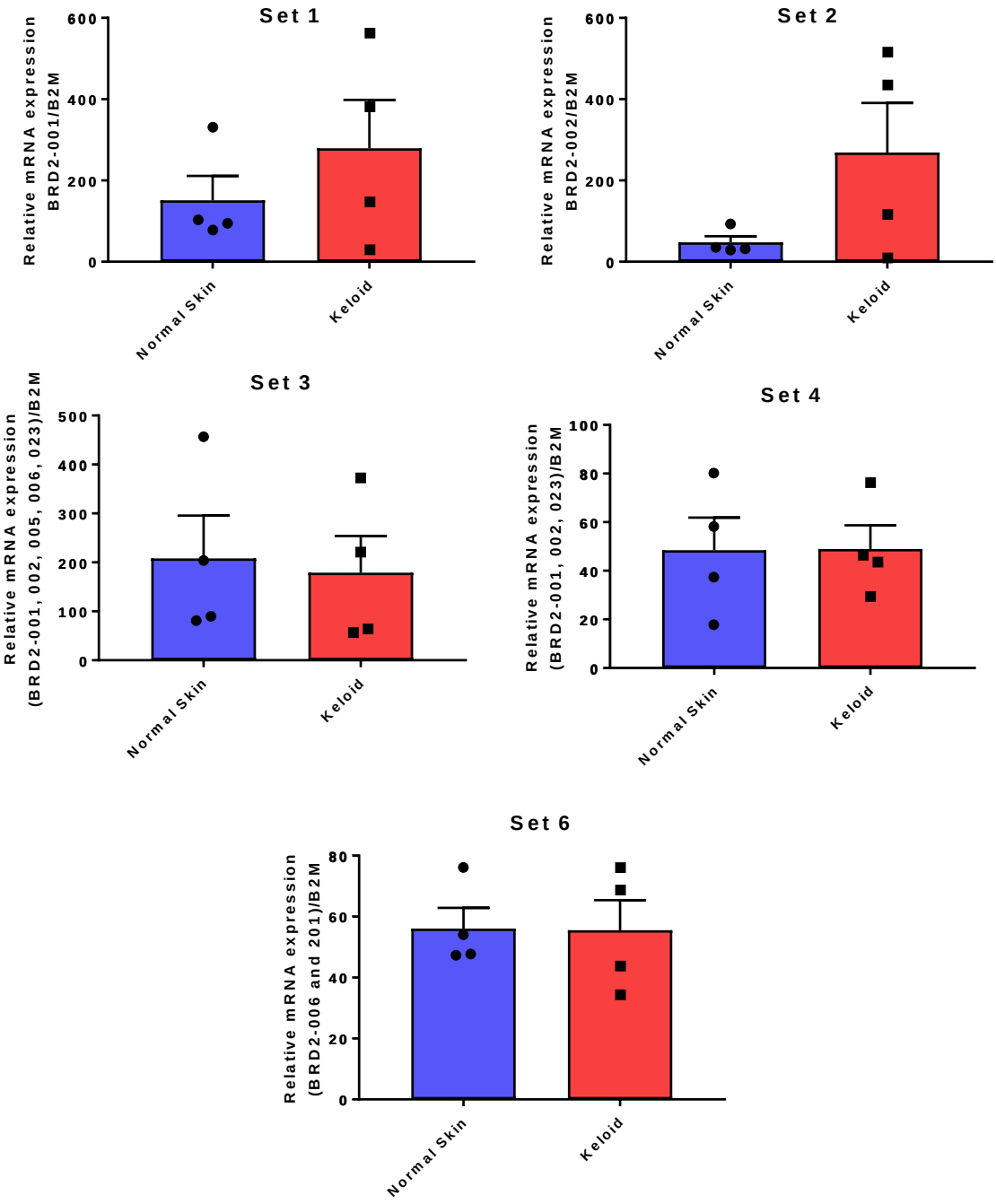



***Figure* 7.5 - Preliminary BRD2 isoform qPCR results on tissue RNA.** qPCR was carried out on n=4 normal skin and keloid scar tissue mRNAs for the best 3 selected primers pairs for each set (denoted in bold in the previous table of primers), primers were selected on the basis of the highest $R^2$ and consistent Ct value when run against a standard curve of pooled mRNA. qPCR was carried out in a 384 well plate format in a 10µl reaction as previously described in Materials & Methods, and the best primer pair out of the 3 was chosen for each set based on the highest $R^2$ and reaction efficiency (calculated from the slope of the standard curve). As previously, qPCR relative values were calculated against a standard curve and normalised by dividing by the relative value for the reference gene B2M.